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Phosphodiesterases Inhibition by Bacilli Calmette-Guérin Contributes to Decrease Asthma in Allergic Rats

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ABSTRACT

Phosphodiesterases (PDE) hydrolyse intracellular cAMP and cGMP to inactive 5' monophosphates. Decreased level of cAMP is involved in the pathogenesis of asthma. We and others have shown that phosphodiesterases were upregulated in the lung of allergic rats, and Bacilli Calmette-Guérin (BCG) induced the production of cAMP *in vitro*. However, it is unclear how BCG's effect asthma and whether it is related to PDEs.

In this study, BCG was intraperitoneally injected into male Sprague-Dawley rats sensitized and later the rats were challenged with ovabumin/pertusis. The inflammation in lungs was measured. Airway hyperresponsiveness was determined using MedLab software after intravenous methacholine challenge. Furthermore, cAMP level and adenylate cyclase activity in lungs were analyzed by ELISA, phosphodiesterases activities were analyzed by HPLC, while PDEs mRNA levels in lungs was analyzed by reverse transcription-polymerase chain reaction. Administration of BCG significantly attenuated allergen-induced lung inflammatory response and hyper responsiveness as compared with vehicle treatment. Furthermore, the levels of cAMP in lungs were significantly increased in BCG-treated allergic rats. Interestingly, administration of BCG decreased the activity of cAMP-PDE, but not adenylyl cyclase (AC), activity in lungs of animals. Furthermore, pretreatment with BCG significantly decreased the mRNA levels of PDE4A, 4C, 5 and 8, which were induced in lungs of allergic rats.

BCG administration attenuated airway inflammatory response and bronchial hyper responsiveness in rats, which are the most important symptoms in asthma. The decreased PDEs mRNA and inhibited cAMP-PDE activities by BCG contribute, at least in part, prevention of allergen-induced airway inflammation and asthma in rats.

Key words: Adenylyl Cyclase; Asthma; Bacillus Calmette-Guérin; cAMP; Phosphodiesterase

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INTRODUCTION

Asthma is a chronic inflammatory condition that causes the airways to constrict and produce excess

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mucus, making breathing difficult. It is characterized by airway inflammation and hyperresponsiveness, and is associated with significant yet avoidable mortality and morbidity resulting in considerable individual and societal burden.[1,2] The hallmark of asthma is airway inflammation.[3] Airway obstruction in asthma can be severe, leading to life-threatening narrowing and closure of airways.[4] Inflammatory cellular infiltration, particularly by eosinophils (and in some cases, neutrophils), plays an important role in airway inflammation in asthma, and this may be attributed to the production of T helper cell Th2 cytokines.[5] Long-term control medications in patients with asthma mainly include corticosteroids, cromolyn sodium and nedocromil, methylxanthines, leukotriene modifiers, and long-acting beta2-agonists (LABAs). Inhaled corticosteroids (ICS), short- and long-acting β_2-adrenoceptor agonists are now the mainstay therapy for asthma. ICS are the most effective class of controller medication available for treating persistent asthma, and are the evidence-based guideline-recommended first-line treatment for control asthma.[2] ICS is highly effective in suppressing airway inflammation. β2-adrenoceptor agonists act primarily to relax airway smooth muscle by stimulating beta2-receptors, which in turn increase cyclic AMP and produce functional antagonism to bronchoconstriction. However, both ICS and β2-adrenoceptor agonists do not influence the natural history of the disease, even though treatment is started early in childhood.[6,7] Therefore, it is urgent to find new drugs with more efficiency to intervene the development of asthma. Phosphodiesterases (PDEs) are important enzymes in the hydrolysis of cAMP and cGMP to inactivate nucleotides.[8] Inhibition of PDEs elevates the levels of cAMP and cGMP, which regulates a variety of cellular functions including airway smooth muscle relaxation and reduction of cellular inflammation as well as immune responses. Decreased cAMP is a contributing factor in pathogenesis of asthma.[9-11] Therefore, many drugs which can induce cAMP production are used in asthma treatment, e.g. β_2-adrenoceptor agonists and theophylline.[12,13] It has been shown that a large number of extracellular stimuli can induce intracellular cAMP production by activating adenylyl cyclase (AC) or inhibiting PDEs. Therefore, the agents regulate AC and PDEs activities may be beneficial in attenuating the progression of asthma. Indeed, cAMP-elevating agents, such as PDE4 inhibitor, significantly decrease airway inflammation and hyperresponsiveness via inhibiting Th2 cell function and pro-inflammatory mediators release.[14-17] Bacillus Calmette-Guérin (or Bacille Calmette-Guérin, BCG) is a bacterial vaccine against tuberculosis that is prepared from a strain of the attenuated live bovine tuberculosis bacillus, Mycobacterium bovis, that has lost its virulence in humans. However, the bacilli have retained enough strong antigenicity to become a somewhat effective vaccine for the prevention of human tuberculosis. There is increasing, but still inconsistent evidence that BCG vaccinations is associated with the normal maturation of the immune system.[18,19] Furthermore, the positive tuberculin reactivity was inversely associated with the prevalence of wheeze, asthma and atopic eczema, especially among Japanese children without a parental allergic history.[20] These findings support the hygiene hypothesis of asthma[21] (e.g., the beneficial effects against children infections and BCG vaccinations on asthma).[22-24] Although there is no comparison between the prevalence of asthma in countries which inoculate BCG routinely and that in countries which do not, indeed, BCG is a strong inducer of T-helper type 1 immune response (Th1) and modulates the development of asthma both in animal models and human beings.[25] BCG infection is known to induce various cytokines, and to inhibit allergen-specific IgE production, ovalbumin (OVA)-induced eosinophilia as well as goblet cell development.[26-29] Accumulating evidence shows that BCG downregulates OVA-induced eosinophilia and specific IgE production via inhibiting Th2 cytokines and enhancing Th1 cytokines.[30,31] BCG could inhibit methacholine (MCh) induced airway hyperresponsiveness (AHR) in mice[32,33] and inhibit airway responsiveness.[34,35] Nevertheless, the molecular mechanism in BCG for attenuating allergens-induced airway inflammatory response is unknown. Recent study showed that BCG induced the production of cAMP, and the regulation of IL-6 by BCG is via a cAMP-dependent pathway in human bladder cancer cells.[36] Therefore, we hypothesize that BCG protects against allergen-induced asthmatic response in rats via a cAMP-dependent pathway, that is, by regulating PDEs. To test this hypothesis, BCG was intraperitoneally administrated to OVA-sensitized and -challenged Sprague-Dawley (SD) rats, and the airway inflammation and airway hyperresponsiveness were determined. We also investigated the effect of BCG on cAMP levels, AC activity, PDE activity as

well as PDEs mRNA levels in ovalbumin (OVA)-challenged asthmatic model of rats.

METERIALS AND METHODS

Allergen Sensitization and Challenge in Rats

Six-week-old male Sprague-Dawley (SD) rats were purchased from Laboratory Animal Center of Zhejiang Chinese Medicine University (Hangzhou, China). All animals were housed in a room maintained at 23 ± 2 °C with 50% ± 10% humidity and a 12 h light 12 h dark cycle (lights on from 8:00 a.m. to 8:00 p.m.). Animals were allowed free access to tap water and regular rodent chow. Rodent chow was withheld for 8 h before the experiments. All the animal care and handling procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University. Rats were sensitized with a subcutaneous injection (1 ml/rat) of a saline suspension containing 0.2% OVA (Sigma, St. Louis, MO) and 10% aluminum hydroxide at their footpad, neck, back, groin, and abdomen on day 0 according to the previous publications.[37,38] At same day, each animal was primed with an intramuscular injection of 2×10^{10} heat-inactivated *bordatella pertussis* organisms (Biological Institute of Shanghai, Shanghai, China). From day 14 after sensitization, animals were challenged once a day for 7 days by exposure for 30 minutes to aerosolized OVA (1%) in saline generated by a jet nebulizer (Master; Pari GmbH, Starnberg, Germany; medium diameter of produced droplets: 1-5μm) (Figure 1).

Animal Grouping and Treatment

Rats were divided into four groups with 6 rats in each group. Control group animals were sensitized and challenged with saline; model group animals were sensitized with OVA and pertussis, and challenged with OVA; BCG group animals were sensitized and challenged as the same to model group, but were administered also with BCG (3×10^6 U/rat) (D2-BP302 strain, (Biological Institute of Shanghai, Shanghai, China) intraperitoneally once a day at days 0 and days 5, as described previously.[39] In dexamethasone-treated animals, allergen sensitization and challenge were the same as that in model group, but dexamethasone (Zhejiang Xianju Pharmaceuticals Co., LTD, Hangzhou, China) was administered intraperitoneally once a day at day 0 and day 5 with a dose of 1 mg/kg. The doses of BCG and dexamethasone were adopted in the present study based on our preliminary results and previous studies.[37,38] We chose dexamethasone as control because bronchial asthma, including its mild forms, are chronic inflammatory diseases of bronchial tubes, requiring application of anti-inflammatory therapy, foremost the use corticosteroids, which are capable to improve considerably prognosis for such patients.[40] Dexamethasone was highly effective in suppressing airway inflammation according to our previous study,[37,38] and we wanted to compare the efficacy of BCG together with dexamethasone in inflammation and lung function when we did not know the exact mechanism of BCG. At day 21, animals were sacrificed, and lung tissues were removed and frozen in liquid nitrogen, and then stored at -80 °C, until analysis.

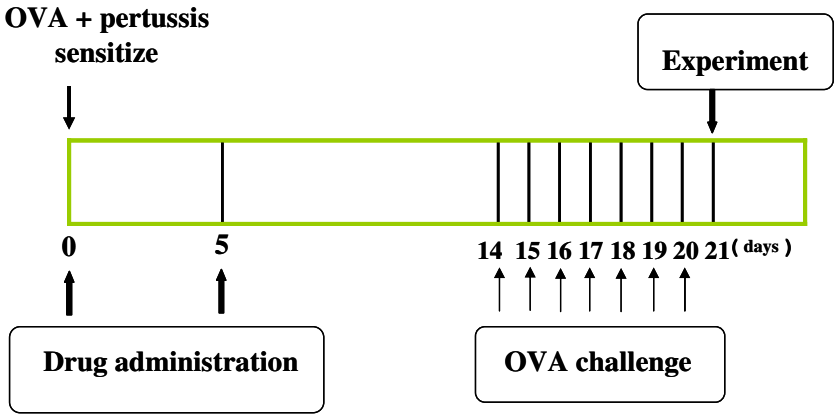

Figure 1. Procedure of asthma model of SD rats.

Preparation of Bronchoalveolar Lavage Fluids (BALF) for Cell Counting and Differentiation

At 24 h after the last OVA challenge, rats were anesthetized with urethane (1 g/kg, i.p.), and lungs were three times lavaged with 5 ml of sterilized normal saline containing 1% bovine serum albumin (BSA) and 5000 IU/l heparin. BALF was combined and centrifuged at 500 ×g at 4 °C for 10 min. The pellets were resuspended with Hank's balanced salt solution (HBSS) for cell counts and classification. Two hundred cells from the cell suspension were stained by Wright-Giemsa and differentiated under light microscope. The results are expressed as the numbers of each type of cell population per one liter of BALF.

Measurement of Airway Hyperresponsiveness

Airway resistance (R_L) and dynamic compliance (C_{dyn}) were determined as changes of airway function after intravenous injection of methacholine (MCh).[41] Briefly, each anesthetized rat was placed supine inside a plexiglas whole-body plethysmograph. The expiratory flow rate was monitored with a fisher tube connected to the airway in a pressure transducer, and the change in lung volume was measured by detecting pressure changes in the plethysmographic chamber through a port in the connecting tube with a pressure transducer. To measure pleural pressure, a needle with a multiholed tip was directly inserted into pleural cavity through a port in the connecting tube with a differential pressure transducer. Transpulmonary pressure was calculated as the difference between mouth and pleural pressure. The signals from all pressure transducers were continuously processed (MedLab, Nanjing Biotech Instruments, Nanjing, China) by fitting flow, volume, and pressure to an equation of motion. Changes in R_L and C_{dyn} from MCh provocation were shown by using a dose-response curve. MCh dosages (0.4g/L to 8g/L, i.v.) were decided from the pre-trial (those could provocate airway hyperreaction in rats and did not cause death). The effects of BCG and dexamethasone were determined by comparing the changes in R_L and C_{dyn} after drug treatment with the mean of MCh response alone in the same rat on previous and successive control periods.

Assay for IL-4 and IFN-γ in Lung Tissue

Fifty milligrams of lung tissues were homogenized in 1 ml of ice-cold 50 mM Tris-Maleate buffer (pH=7.4, containing 5 mM $MgSO_4$). The assay mixture was cooled on ice, followed by centrifugation at 12,000×*g* for 30 minutes at 4 °C. The amount of IL-4 and IFN-γ present in the supernatant was determined using a rat IL-4-specific ELISA kit (KRC0041, Biosource) and a rat IFN-γ-specific ELISA kit (BMS621, Bender MedSystems), respectively. For IL-4 assay kit, the inter-assay and intra-assay coefficients of variation were 7.8% and 2.9%, respectively. For IFN-γ assay kit, the inter-assay and intra-assay coefficients of variation were 9.0% and 8.0%, respectively.

Histological Examination

The lungs were immersed in 10% neutral formalin for 7 days. After tissues were paraffinized, 5 μm sections were prepared and stained with hematoxylin-eosin for observation of inflammatory cell infiltration under light microscope.

Assay for cAMP in Lung Tissue

Fifty milligrams of lung tissues were homogenized in 1 ml of ice-cold 50 mM Tris-Maleate buffer (pH= 7.4, containing 5 mM $MgSO_4$), the assay mixture was cooled on ice, followed by centrifugation at 12,000×*g* for 30 minutes at 4 °C. The amount of cAMP present in the supernatant was determined by cAMP ELISA kit (R&D Systems, Inc., Minneapolis, USA). For this cAMP assay kit, the inter-assay and intra-assay coefficients of variation were 7.6% and 4.2%, respectively.

Assay for AC Activity

AC activity was measured as described previously.[42] Briefly, the AC assay mixture (500 μl) contained 50 mM Tris-Maleate buffer, 8 mM theophylline, 4 mM DTT, 1 mM EGTA, and 1 mM ATP. The AC reaction was started by the addition of 100 μl lung homogenate into 500 μl of AC assay mixture, and was performed at 30 °C for 15 minutes. The reaction was stopped by boiling the mixture for 3 minutes. The assay mixture was cooled on ice, followed by centrifugation at 4 °C. The amount of cAMP present in the supernatant was determined by cAMP ELISA kit (R&D).

Assay for cAMP-PDE Activity

Twenty-five milligrams of lung tissues were homogenized in 1 ml of ice-cold 30 mM N-2-hydroxyethylpiperazine- N'-ethane sulfonic acid (pH =7.4) containing 0.1% Trion X-100 (Sigma). The cAMP-PDE assay mixture (200 μl) in phosphate-buffered saline (pH= 7.4) contained 137 mM NaCl, 2.7

mM KCl, 8.8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 μM cAMP (Sigma). The PDE reaction was started by the addition of 20 μl lung homogenate into 200 μl of cAMP-PDE assay mixture, and was performed at 37°C for 30minutes. The reaction was stopped by boiling the mixture for 3 minutes. The assay mixture was cooled on ice, followed by centrifugation at 12,850×*g* for 30 minutes at 4 °C. The amount of cAMP present in the supernatant was determined by HPLC (Agilent TC-C18 4.6×250 mm; Hewlett-Packard, Palo Alto, CA) by using a standard curve of cAMP.[38]

Assay for PDEs mRNA Expressions in Rat Lungs

Total RNA was isolated from each tissue using Trizol reagent (Sigma). The preparation of first-strand complementary DNA (cDNA) from total RNA was performed using a first-strand cDNA synthesis kit (Shanghai Sangon Biological Engineering Technology and Service Co. Ltd., Shanghai, China). Sequences of polymerase chain reaction (PCR) primer sets used for PDEs and internal standard. β-actin were described previously and listed in table 1,[43-46] PCR amplification was performed in a reaction buffer (10 mM Tris-HCl, pH 9.0; 100 mM KCl; 80 mM $[NH4]_2SO_4$; and 0.1% Nonidet P-40) containing 0.2 mM each nucleotide, 1.5 mM $MgCl_2$, 500 nM each primer, and 1 U of Taq DNA polymerase (Sangon) in a total volume of 25 μl, and PCR was performed for 35 cycles. After amplification, 8 μl of each reaction mixture was resolved by electrophoresis on a 1.5% agarose gel containing ethidium bromide, and the PCR product bands were quantified by using the UVP Gel Documentation system (UVP, Upland, CA). The levels of PDEs mRNAs were calculated relative to β-actin.

Assay for Total Protein

The amounts of total protein were determined by the Bradford method using a coomassie blue staining protein assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Bovine serum albumin was used as the standard.

Statistical Analysis

Data are expressed as means ± SD. Statistical analysis was performed with a one-way ANOVA to evaluate the levels of cAMP-PDE activity, AC activity, cAMP, IL-4 and IFN-γ, a nonparametric test to evaluate pulmonary functions, and a student two-tailed t-test to calculate the inflammatory cells and evaluate the PDE4 mRNA expression. Significance was assessed at the $P < 0.05$ level. Experiments were performed independently for at least twice. Results were qualitatively identical, and representative results are shown.

RESULTS

Effect of BCG on Allergen-induced Inflammatory Cell Infiltration in Lungs

Male SD rats were sensitized and challenged with OVA to induce lung inflammation. Lungs were lavaged at 24 h post-last OVA challenge, and differential cells in BALF were counted to investigate the effect of BCG on inflammatory cells influx. We found that the total number of cells in BALF of OVA-vehicle rats was 5-fold greater than that in NS-vehicle rats (NS control: 35.0×10^8 cells/L). BCG treatment significantly decreased the number of total cells in BALF, which was slightly more than that in dexamethasone-treated rats (Figure 2A).

Table 1. Primer sequences used in this study

Gene	Primer sequences(5'-3')	Length of PCR product (bp)	Tm (°C)
β-actin	Sense: AACCCTAAGGCCAACCGTGAAAAG Antisense: GCTCGAAGTCTAGGGCAACATA	343	57
PDE4A	Sense: TCAACACCAATTCGGAGCTGG Antisense: GTCTTCAGGTCAGCCAGGAGG	216	61
PDE4C	Sense: ACTGAGTCTGCGCAGGATGG Antisense: ACTCCTCTTCCTCTGCTCTCCTC	539	62
PDE5	Sense: CTGTCTGATCTGGAAACAGC Antisense: GCAATCAGCAATGCAAGCGT	250	57
PDE8	Sense: GGAGAACCAACTCCTTCCTGTG Antisense: AGGCATCCCATGCATCAAAC	592	58

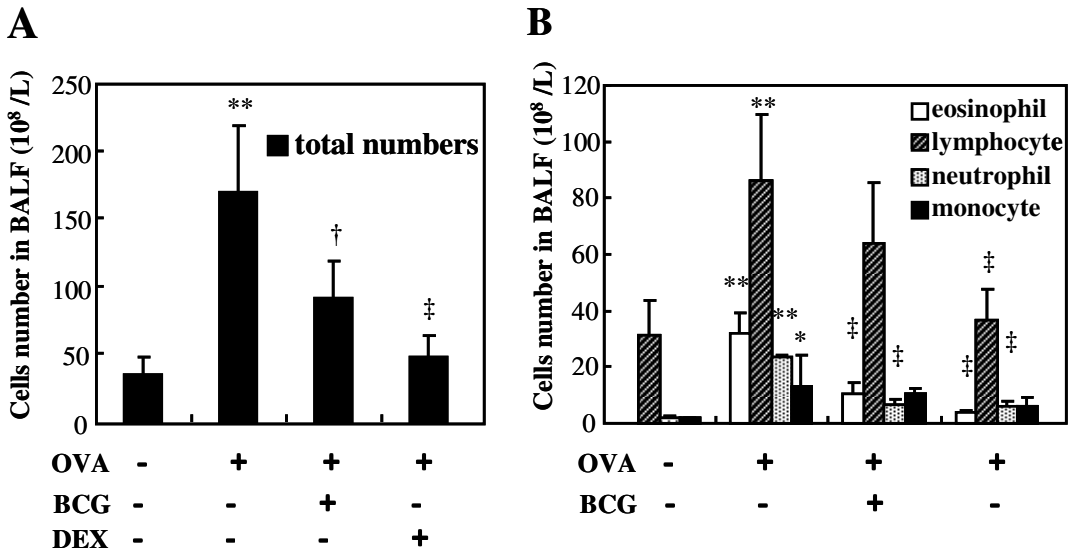

Figure 2. Effects of BCG and dexamethasone on total cells and differential cell counts in BALF of allergic rats. Rats were sensitized and challenged as described in Methods, and BALF was harvested 24 h post-last OVA challenge. Total inflammatory cell numbers in BALF were counted (A), and differential cell counts was performed on a minimum of 200 cells to classify eosinophils, neutrophils, lymphocytes and monocytes (B). Data are expressed as mean ± SD. *: $p<0.05$, **: $p<0.01$, versus NS-challenged and vehicle-treated rats ($n=6$, NS-vehicle group); †: $p<0.05$, ‡: $p<0.01$ versus OVA-challenged and vehicle-treated rats ($n=6$, OVA-vehicle group).

The numbers of eosinophils, neutrophils, lymphocytes and monocytes/macrophages in the BALF of OVA-vehicle rats were increased 31-, 11-, 3- and 8-fold, respectively, as compared with those in NS-vehicle rats (e.g., the numbers of eosinophils, neutrophils, lymphocytes and monocytes/macrophages were 0.12×10^8 cells/L, 1.90×10^8 cells/L, 31.3×10^8 cells/L and 1.64×10^8 cells/L in NS-vehicle group) (Figure 2B). Treatment with BCG significantly decreased the number of eosinophil and neutrophil numbers in BALF as compared to allergic rats with vehicle treatment (Figure 2B). These results suggest that BCG inhibits inflammatory cells influx in lungs in response to allergen sensitization and challenge in rats.

Effect of BCG on Airway Inflammation

Lung tissue was harvested at 24 h post-last ovalbumin challenge. As shown in figure 3, OVA sensitization and challenge (Figure 3B) induced significant infiltration of inflammatory cells, especially eosinophils, into the peribronchiolar and perivascular tissues as compared with control rats (Figure 3A). Treatment with either BCG (Figure 3C) or dexamethasone (Figure 3D) significantly attenuated OVA-induced eosinophil-rich leukocyte infiltration in lungs as compared with vehicle treatment.

Effect of BCG on Th1/Th2 level in Lung of Allergic Rats

It has been shown that the ratio of Th1/Th2 cytokines was decreased in asthma, and IFN-γ and IL-4 are critical cytokines secreted from Th1 and Th2 cells, respectively. Therefore, the levels of IL-4 and IFN-γ in the lungs of rats from the various groups were measured (Figure 4A and Figure 4B). OVA sensitization and challenge decreased IFN-γ and significantly increased IL-4 production in rat lung. Treatment with BCG (3×10^6 U/rat) or dexamethasone (1mg/kg) completely increased IFN-γ and suppressed the increased IL-4 production in response to allergen sensitization and challenge.

Effect of BCG on Allergen-induced Airway Hyperresponsiveness in Rats

In the rat model of allergic asthma, OVA sensitization caused a significant decrease in C_{dyn} whereas R_L was significantly increased in allergic rats as compared to control rats (Figure 5).

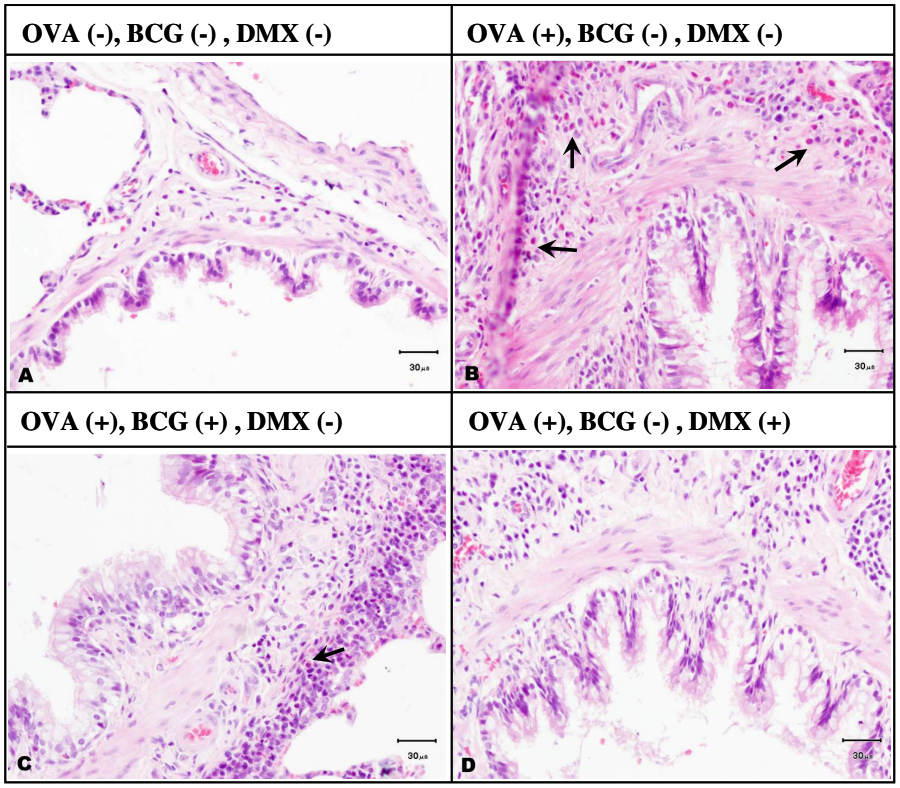

Figure 3. Effects of BCG and dexamethasone on allergen-induced inflammation cells infiltration in lungs. Rats were sensitized and challenged as described in Methods. Lung tissues were harvested 24 h after the last OVA challenge and immersed in 10% neutral formalin for 7 days. After tissues were paraffinized, 5 µm sections were prepared and stained with hematoxylin-eosin for observation of cell infiltration under light microscope. Arrow: pointing to eosinophils

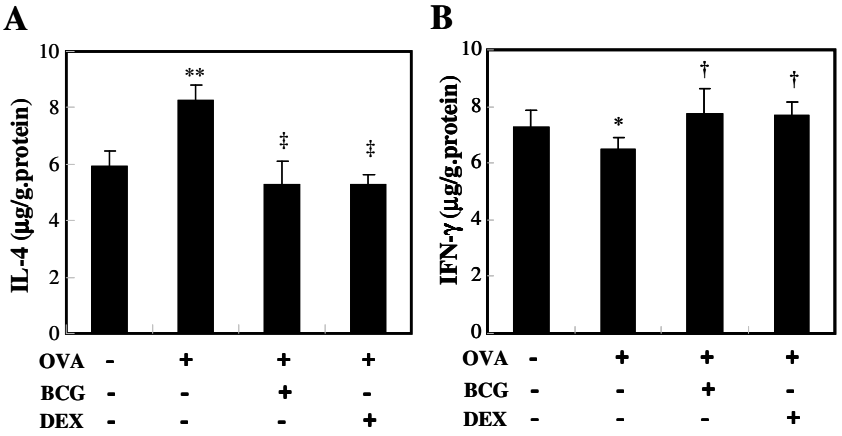

Figure 4. Effect of BCG and dexamethasone on IL-4 and IFN-γ levels in lungs of allergen challenged rats. Rats were sensitized and challenged as described in Methods, lung tissues were harvested 24 h after the last OVA challenge and used for determining the level of IL-4 (A) and IFN-γ (B) using a rat IL-4-specific ELISA kit and a rat IFN-γ-specific ELISA kit.. Data are expressed as mean±SD. *: $p<0.05$, **: $p<0.01$, versus NS-challenged and vehicle-treated rats ($n=6$, NS-vehicle group); †: $p<0.05$, ‡: $p<0.01$ versus OVA-challenged and vehicle-treated rats ($n=6$, OVA-vehicle group).

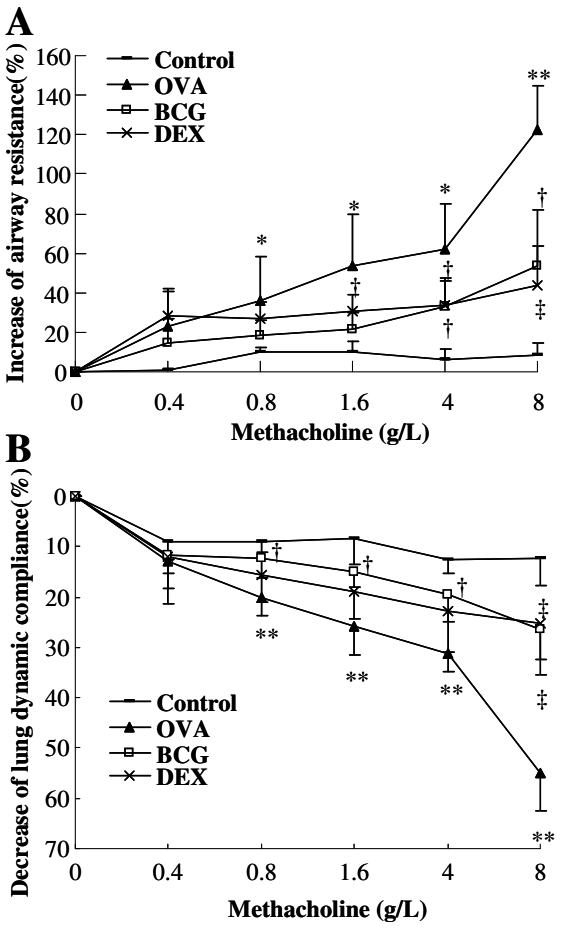

Figure 5. Effects of BCG and dexamethasone on methacholine-induced change of lung resistance (RL) and lung compliance (Cdyn) in allergen challenged rats. Rats were sensitized and challenged as described in Methods, 24 h post-last OVA challenge, R_L (A)and C_{dyn} (B)against aerosol methacholine was determined as described in Methods. Data are expressed as mean±SD. *: $p<0.05$, **: $p<0.01$, versus NS-challenged and vehicle-treated rats ($n=6$, NS-vehicle group); †: $p<0.05$, ‡: $p<0.01$ versus OVA-challenged and vehicle-treated rats ($n=6$, OVA-vehicle group).

Treatment with BCG significantly improved airway hyperresponsiveness including both C_{dyn} and R_L caused by sensitization and challenge at 8 $g \cdot L^{-1}$ MCh ($p<0.05$), and this effect was almost the same as that of dexamethasone treatment (Figure 5). These results suggest that the improvement of airway hyperresponsiveness by BCG may be due to decreased inflammatory response induced by OVA sensitization and challenge in lungs.

Effects of BCG on cAMP Level in Lung of Allergic Rats

It has been shown that decreased level of cAMP plays an important role in the pathogenesis of asthma, and BCG induced cAMP levels in bladder cancer cells.[31,38,47] We hypothesize that BCG treatment could increase the cAMP level therefore attenuating airway inflammation and hyperresponsiveness. As expected, the level of cAMP was significantly decreased in lungs of allergen-induced asthmatic rats as compared to that in control animals (Figure 6). Administration of BCG increased the level of cAMP in lungs as compared to model group. Dexamethasone could also increase the level of cAMP in lungs, but its effect on cAMP was slightly weaker than that by BCG (Figure 6).

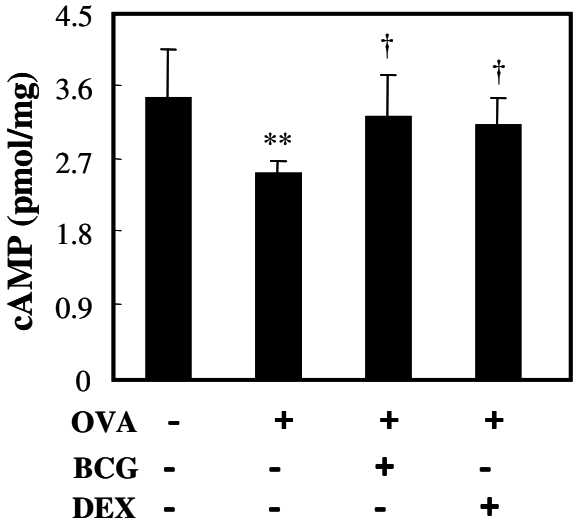

Figure 6. Effects of BCG and dexamethasone on cAMP production levels in lungs of allergic rats. Rats were sensitized and challenged as described in Methods, lung tissues were harvested 24 h after the last OVA challenge and used for determining the level of cAMP using a cAMP ELISA kit. Data are expressed as mean±SD. **$p <$ 0.01 versus NS-challenged and vehicle-treated rats (n=6); †$p <$ 0.05 versus OVA-challenged and vehicle-treated rats (n=6).

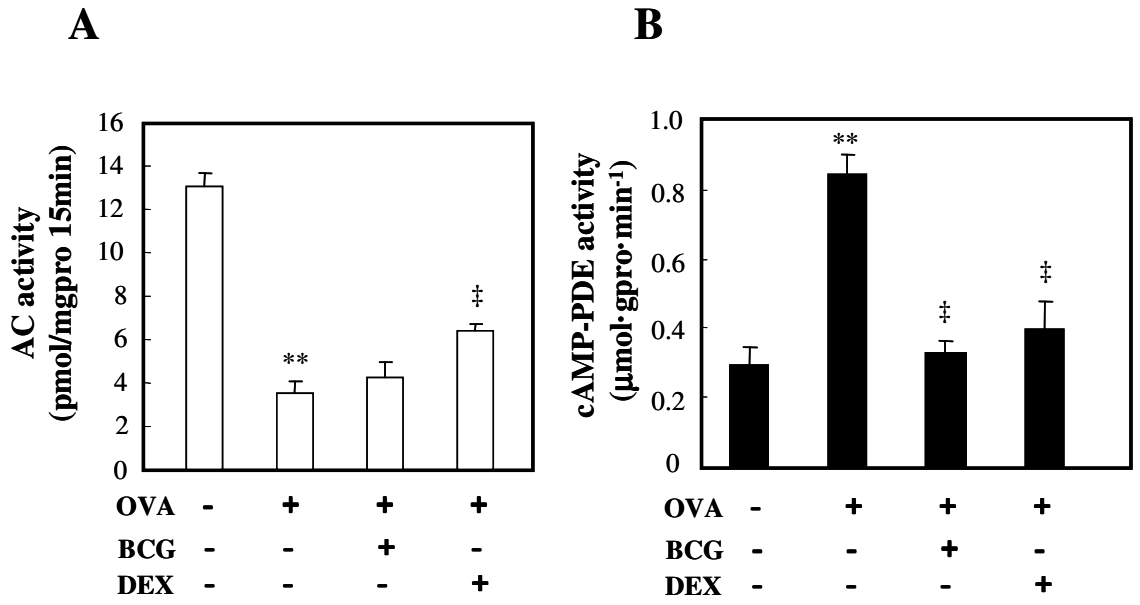

Figure 7. Effects of BCG and dexamethasone on AC activity and cAMP-PDE activities in lungs of allergic rats. Rats were sensitized and challenged as described in Methods, lung tissues were harvested 24 h post-last OVA challenge and used for determination of AC activity by an AC reaction and cAMP ELISA kit (A), and for determination of cAMP-PDE activity by HPLC (B), respectively. Data are expressed as mean ± SD. *****p* **< 0.01 versus NS-challenged and vehicle-treated rats (n=6); ‡*p* < 0.01 versus OVA-challenged and vehicle-treated rats (n=6).**

Effects of BCG on AC and PDE Activity in Lung of Allergic Rats

To investigate whether the change of cAMP level is related to the alteration of AC and/or PDEs activities. We determined the activities of AC and cAMP-PDE in lungs of control and allergen induced asthmatic rats. OVA sensitization and challenge significantly inhibited AC activity in lungs. However, treatment with BCG did not result in any obvious change in AC activity as compared to the vehicle treatment in allergen induced asthmatic rats. Interestingly, dexamethasone could significantly increase AC activity, and its effect on AC was stronger than BCG (Figure 7A).

Furthermore, pretreatment with BCG significantly attenuated the OVA-induced increase in cAMP-PDE activity in rat lungs, and its effect on cAMP-PDE activity was even more effective than dexamethasone (Figure 7B).

Effects of BCG on PDEs mRNA Expression in Lung of Allergic Rats

The mRNA levels of PDEs in lungs of control and allergic rats were determined in the absence or presence of BCG pretreatment. It was found that the mRNA levels of PDE1, PDE2, PDE3, PDE4A, PDE4B, PDE4C, PDE4D, PDE5, PDE7, PDE8, PDE9, PDE10, and PDE11 were expressed in lungs of control rats although the expression level of PDE10 mRNA was low (data not shown). OVA sensitization and challenge significantly increased the mRNA levels of PDE4A, PDE4C, PDE5, and PDE8 as compared to control rats (Figure 8A-D). Treatment with BCG significantly inhibited the mRNA expression level of PDE4A, 4C, 5 and 8 in lungs of allergen induced asthmatic rats as compared to that of model rats (Figure 8A-D). Similarly, dexamethasone showed significant effect on inhibition of PDE4A, 4C, 5 and 8 mRNA expression induced by allergen sensitization and challenge in lungs as compared to the model group (Figure 8A-D).

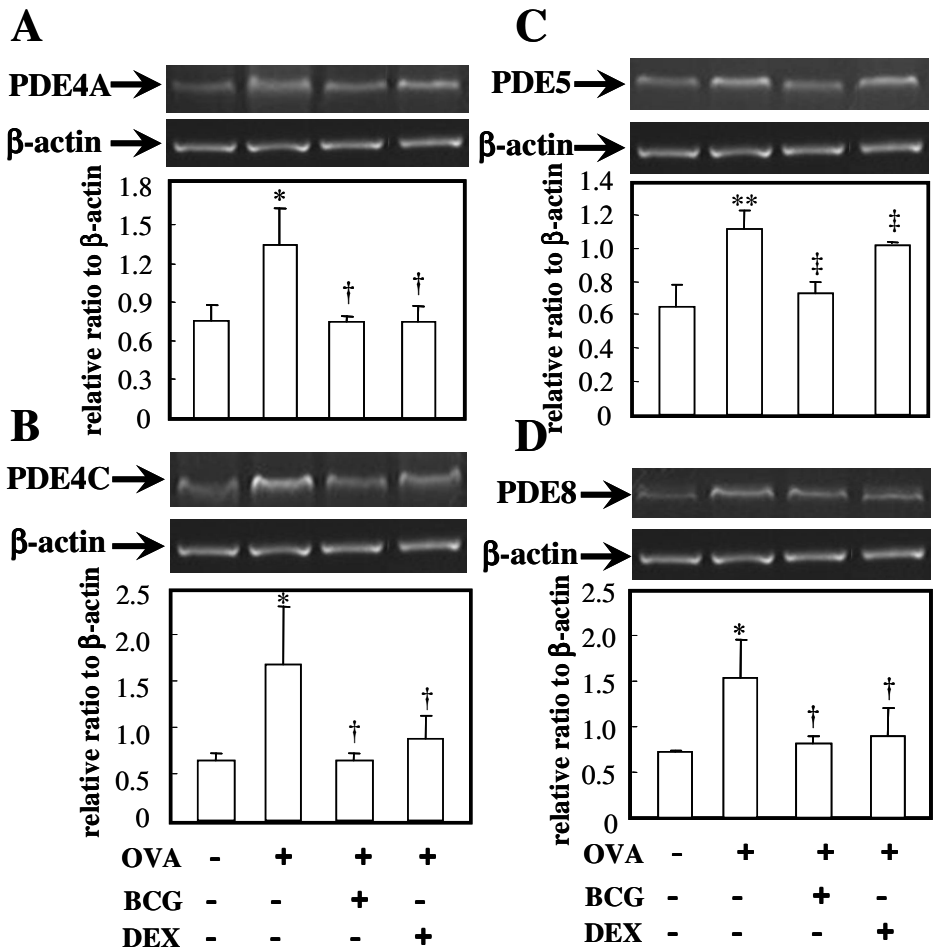

Figure 8. Effects of BCG and dexamethasone on PDEs mRNA expression in lungs of allergic rats. Rats were sensitized and challenged as described in Methods, lung tissues were harvested 24 h post-last OVA challenge and used for measurement of PDEs mRNA expression by RT-PCR. RNA isolation and cDNA synthesis were performed as described in Methods. The representative bands derived from PCR products of each PDE subtype are shown, and the intensity of PCR product-derived bands was quantified. The expression of PDE4A(A), 4C(B), 5(C), 8(D) were normalized to β-actin, and the results are expressed as means ± SD from six rats. *$p < 0.05$, **$p < 0.01$ versus NS-challenged and vehicle-treated rats (n=6); †$p < 0.05$, ‡$p < 0.01$ versus OVA-challenged and vehicle-treated rats (n=6)

DISCUSSION

In recent decades, there has been a significant increase in the prevalence of asthma in various populations. Inhaled corticosteroids (ICS), short- and long-acting β_2-adrenoceptor agonists are the current mainstay therapy for asthma. However, β_2-adrenoceptor agonists only alleviate the symptoms. ICS are highly effective in suppressing airway inflammation, but they do not influence the natural history of the disease even if treatment had been started early in childhood.[6,7] Moreover, ICS can produce many side effects[48] and are largely ineffective in virus-induced exacerbations[49] and in those asthmatics who smoke.[50] Therefore, it is urgent to develop new drugs with more efficiency and less side effects in asthma treatment. BCG is originally known as a vaccine for tuberculosis, and regulates various cytokines production, as well as inhibit allergen-specific IgE production, OVA-induced eosinophilia, and goblet cell

development.[30,31] Therefore, our rationale in this study was that BCG is beneficial to asthma treatment. It has been shown that a relatively high dose of BCG infection inhibits airway sensitivity, even after allergen sensitization.[39] Indeed, this was corroborated by our results showing decreased airway inflammatory response and airway hyperresponsiveness in BCG-treated and OVA-induced asthmatic model of SD rats. This was consistent with previous studies showing OVA-induced eosinophilia, goblet cell hyperplasia, high level of Th2 cytokines and airway hyperresponsiveness were inhibited by BCG.[31,51-54]

It has been shown that the level of cAMP in lungs was decreased in asthmatics.[38,47] We, and others, have found that some cAMP-inducing reagents, e.g. PDE inhibitors, theophylline, β_2 agonists, were effective in attenuating lung inflammatory response and improving lung function in patients with asthma and asthmatic model of animals.[55-57] In the present study, we also found that treatment of BCG increased the level of cAMP in lungs induced by OVA, suggesting the protective effect of BCG on OVA-induced asthma in rats due to elevation of cAMP. This is consistent with the previous study showing an increased cAMP in human bladder cancer cells in response to BCG incubation.[36] It is well known that the level of cAMP is regulated by the AC and cAMP-PDE. Thus, we determined both enzymes activities in lungs of allergen-induced asthmatic rats in the absence or presence of BCG. We found that the AC activity was decreased, whereas an increased level of cAMP-PDE activity was observed in lungs of allergen-induced asthmatic rats. Furthermore, treatment with BCG decreased cAMP-PDE activity in lungs of allergic rats. It is interesting to note that the AC activity was inhibited in OVA-induced asthma rats, which was not affected by BCG. In this study, we also found that the effect of dexmethasone on cAMP was slightly weaker than BCG, whereas its effect on AC was stronger than BCG and its effect on cAMP-PDE activity was weaker than BCG. These results indicate that BCG and dexamethasone may have different mechanisms although they showed the same effect of increasing cAMP in lungs.

PDEs superfamily comprises 11 biochemically and pharmacologically distinct enzyme families (PDE1-11), which can hydrolyze cAMP and/or cGMP. It has been shown that PDE3, 4, 7, 8 are cAMP specific, PDE1, 5, 6, 9 are cGMP specific, and PDE2, 10, 11 can hydrolize both cAMP and cGMP. PDE inhibitors, especially PDE4 inhibitors, have been shown to attenuate airway inflammation and hyperresponsiveness in rodent models of asthma.[58,59] Indeed, some PDE4 inhibitors, such as roflumilast and cilomilast, had successfully translated in phase II and III clinical trials and roflumilast (Daxas; Nycomed) had been granted marketing authorization by the European Commission for the maintenance treatment of severe chronic obstructive pulmonary disease, as an add-on to bronchodilator treatment in July 2010.[60,61] We found that the OVA sensitization and challenge significantly increased the expression level of PDE4A, PDE4C, PDE4D, PDE5, PDE7 and PDE8 mRNA as compared to control rats (data not shown), which was consistent with previous studies indicating that PDEs activation play an important role in the pathogenesis and development of asthma.[38] Treatment of BCG inhibited PDE4A, PDE4C, PDE5 and PDE8 mRNA expressions, which may contribute to the decreased cAMP-PDE activity. The down-regulated PDEs by BCG attenuate cAMP degradation thereby increasing cAMP levels in lungs, which may be one of the mechanisms for attenuating airway inflammation and hyperresponsiveness in response to allergens exposure.

It has been shown that inflammation in asthma is correlated with the activation of PDE4A and PDE4B, and PDE4A is important in pathogenesis and development of asthma.[62,63] Interestingly, PDE4C mRNA expression was increased in T-lymphocytes of asthma patients and in allergic rats although it appears to be more distal from other PDE4 subfamilies with certain key residues being disordered.[37,64] Other PDE families, such as PDE5 and PDE8, have also been shown to play an important role in the pathogenesis of asthma, and their inhibitors have a potential in attenuating the progress of this disease.[65-67] Indeed, the mRNA levels of all these PDE4A, PDE4C, PDE5 and PDE8 were significantly increased in asthmatic rats, which were lowered by BCG. Importantly, no effect of BCG on PDE4D that appears to mediate nausea and vomiting by PDE4 inhibitor was observed in this study. Hence, BCG could inhibit several PDEs with less side-effect than single PDE4 inhibitor, which could be more beneficial in asthma treatment. Furthermore, inhibition of other PDEs classes, along with PDE4 by BCG may be one of the effective avenues in controlling the progression of asthma because a mixed PDEs inhibitor provides more bronchodilator and bronchoprotective

effect in addition to the beneficial effects by PDE4 inhibitor.[55]

In conclusion, BCG pretreatment significantly attenuated allergen-induced airway inflammation and hyperresponsiveness in rats. The mechanism underlying these findings is associated with the increased level of cAMP via inhibiting certain PDEs mRNA expression and cAMP-PDE activity in rat lung.

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