

BCG Infection in Allergen-Presensitized Rats Suppresses Th2 Immune Response and Prevents the Development of Allergic Asthmatic Reaction

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Recent investigations demonstrate that bacille Calmette-Guérin (BCG), a potent inducer of Th1 response, infection prior to allergen sensitization inhibits Th2 immune responses to the allergen. However, it is not clear whether BCG infection in allergen-presensitized rats switches off Th2 response and prevents allergic asthmatic reaction to the subsequent allergen exposure. In this study we investigate whether BCG infection in ovalbumin (OVA)-presensitized Sprague-Dawley rats suppresses airway hyperresponsiveness and eosinophilic inflammation induced by OVA and Th2 cytokine production. BCG infection in OVA-presensitized rats significantly inhibited not only the sensitivity of airway smooth muscle to electrical field stimulation and acetylcholine but also absolute eosinophil counts in bronchoalveolar lavage fluid. As a correlate, interleukin-4 (IL-4) production significantly decreased and interferon- γ (IFN- γ) slightly increased, resulting in a markedly decreased ratio of IL-4-IFN- γ in OVA-presensitized rats with BCG infection. These results indicate that BCG infection in pre-sensitized rats suppresses allergic asthmatic reaction and Th2 immune response. It is possible from these findings that BCG vaccine may be used as an immunomodulating agent for the sensitized host with preestablished Th2 memory.

KEY WORDS: BCG vaccine; asthma; cytokines.

INTRODUCTION

Immunoglobulin-E (IgE)-mediated allergic disorders, in particular allergic asthma, are mediated primarily by Th2 lymphocytes (1). Th1 and Th2 cells reciprocally regulate each other through the cytokines they secrete. For example, interferon- γ (IFN- γ) and interleukin-12 (IL-12)

inhibit Th2 cells and IL-4 and IL-10 down-regulate the activity of Th1 cells (1, 2). Association of allergic disorders with the Th2 cytokine pattern raises the possibility to design prophylactic and therapeutic modalities in a way that would promote a Th1 immune response and thereby down-regulate the Th2 immune response (2, 3).

Humans are initially exposed to environmental allergens during infancy or early childhood and there is a growing consensus that high-level allergen exposure during the first few months of life predisposes to allergic sensitization (4), which manifests in later childhood as Th2 reactivity. Early childhood may be considered as an opportunity to regulate the development of normal anti-allergen immunity in unsensitized children. However, development of sensitization to allergens occurs within the first 2 years of life (5). A significant proportion (20–30%) of general populations are sensitized to a few major offending aeroallergens (6). Furthermore, transplacental priming of the T-cell system against environmental allergens appears relatively common (7–9) and this process may intrinsically result in the development of Th2 skewed immunity (10). These Th2 responders constitute the high-risk group for subsequent development of severe airway hyperresponsiveness (AHR), which will progressively worsen in a significant proportion, into adulthood resulting in chronic asthma. Therefore, for the sensitized host with preestablished Th2 memory, immunoprophylactic strategies are needed to switch off atopy and prevent allergic asthmatic reaction to the subsequent allergen exposure.

Mycobacterium species, including bacille Calmette-Guérin (BCG) used as a vaccine to prevent human tuberculosis, are known to be potent inducers of Th1 response (11, 12). Recent studies in mice have demonstrated that mycobacterial infections (13–15) prior to allergen sensitization inhibit Th2 immune responses to the allergen. Wang and Rook (16) also recently reported

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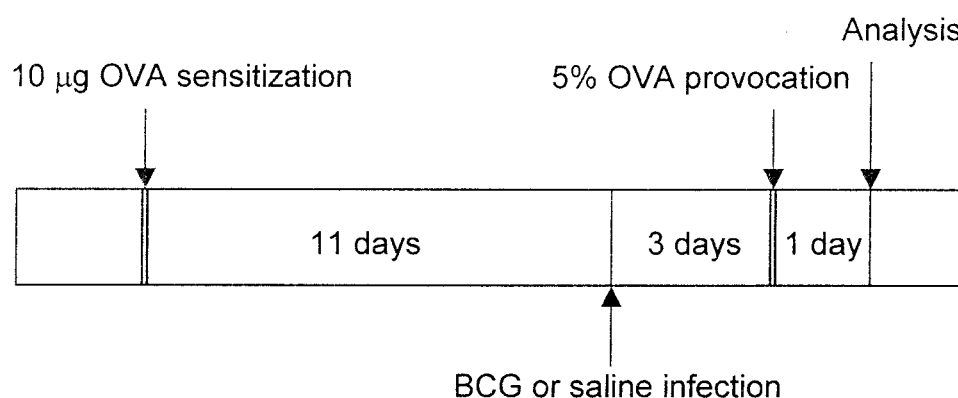


Fig. 1. Schematic representation of the time course of ovalbumin (OVA) sensitization, bacille Calmette–Guérin (BCG) infection, and OVA provocation in our experimental study.

that a single injection of killed *M. vaccae* into allergen-prensensitized mice suppressed serum IgE and allergen-specific IL-5 synthesis.

The purpose of this study was to investigate whether BCG infection in allergen-prensensitized rats inhibited both AHR and eosinophilic inflammation, hallmarks of bronchial asthma, induced by the allergen. As a correlate, allergen-driven cytokine production patterns in BCG-infected rats were examined. The results suggest that BCG infection suppresses airway muscle sensitivity, airway eosinophilia, and Th2 cytokine production in the sensitized rats. It is possible from these findings that BCG vaccine may be used as an immunomodulating agent for the sensitized host with preestablished Th2 memory.

METHODS

Experimental Rats

Eighteen male Sprague-Dawley (SD) rats (Sam-Yook Animal Company Ltd., Korea) were used in this study. They were specific pathogen-free rats that were raised on standard diets in an animal care room at Chonnam National University Medical School until they reached 280 g body weight. They were all sensitized and provoked with ovalbumin (OVA) and 9 rats were infected with BCG (Fig. 1). All procedures were reviewed and approved by the committee on animal research at Chonnam National University Medical School and Chonnam University Hospital.

OVA SENSITIZATION. All 18 SD rats were actively sensitized with a subcutaneous injection of 10 µg OVA (grade III, Sigma) together with 200 mg A1(OH)₃ and 1

ml killed Pertussis vaccine (1×10^{10} /ml, Korea Green Cross Corporation).

BCG INFECTION. Nine rats were infected intraperitoneally with 6×10^5 colony forming units (CFUs) of live attenuated BCG (Pasteur Merieux, France) 11 days after OVA sensitization.

OVA PROVOCATION. Three days after BCG infection, all rats were provoked with 5% OVA aerosols using Pari Boy nebulizer (output = 0.42 g/min, mass median aerodynamic diameters = 4.8 µm, pressure = 0.75 bar) in an exposure chamber (length = 56 cm, width = 44 cm, height = 46 cm) developed in our laboratory.

Bronchoalveolar Lavage (BAL) and Differential Cell Counts

The rats were euthanized by an intraperitoneal injection of 5 mg/kg thiopental sodium and exsanguinated through the inferior vena cava 24 h after the provocation with OVA aerosols. The trachea was cannulated with PE-20 polyethylene tube and BAL was performed by five lavages with 4 ml physiologic saline (4°C). The recovered fluid was immediately centrifuged at 1500 rpm for 10 minutes at 4°C (VS-6000, Vision, Korea). The cell pellet was resuspended with 0.5 ml PBS (pH = 7.4). Total cells were counted under light microscopy (100×) using hemocytometer (Neubauer chamber) after mixing the 50 µl cell pellet solution with 950 µl acetic acid (3%). The remaining cell pellet solution was diluted with PBS to make cell counts 1×10^6 /ml. Cytospins were prepared for each sample by centrifuge of 400 µl cell pellet solution at 700 rpm for 5 minutes at 4°C (CF-120, Sakura, Japan). Two slides for each sample were obtained. After fixations, each slide was stained with

Diff-Quik. Differential cell counts of 500 cells in each slide were performed under light microscopy (400 $\times$) and mean cell counts of the two slides were used for statistical analysis. The cells were classified as either neutrophils, eosinophils, lymphocytes, or macrophages by standard morphological criteria.

Assessment of Airway Smooth Muscle Responsiveness

After BAL was performed, the trachea was immediately removed and was placed in oxygenated fresh Krebs-Henseleit (K-H) solution (115.5 NaCl, 4.16 KCl, 2.5 CaCl₂, 1.16 MgSO₄, 1.6 NaH₂PO₄, 21.9 NaHCO₃, and 11.1 mM glucose). The trachea was trimmed free of fat and connective tissue and cut into four transverse pieces, each containing four to five cartilagenous rings. The epithelium was left intact. Tracheal ring segments were mounted vertically using platinum hooks inserted through the lumen in 10 ml organ baths containing K-H solution (pH = 7.4) bubbled with 95% O₂ and 5% CO₂ at 37°C. The upper hook was fastened to a force transducer (Grass FT03) using silk thread (Ethicon 4-0). Tissues were equilibrated for 1 1/2 hr with washing at least every 20 minutes under a resting tension of 0.75 g. In a preliminary study, the optimal tension for the contractile response of tracheal ring segment of SD rats was found to be 0.75 g. Isometric contractile responses were measured with force transducer and were recorded on a polygraph (Grass 7 series).

During the equilibrium period, the maximal response to electrical field stimulation (EFS) was measured. The lower platinum hook served as one of the poles of the EFS source and the other pole for EFS was not placed in physical contact with the segment; the two poles were 1 cm apart. Pulses were delivered for 10 seconds from an electrical stimulator (Dual Impedance Research Stimulator, Harvard) with a voltage of 50 V. In the preliminary study, the optimal voltage was 50. With a pulse duration of 0.5 msec, 0.5, 1, 2, 5, 10, 20, 50, and 100 Hz were delivered with 1 1/2-minute intervals in progressively increasing pulses. After EFS-induced responses, the tissue was then washed to regain the baseline tension and soaked for 30 minutes. Finally, the cumulative dose-response relationships to acetylcholine (ACh) (0.1, 0.3, 1, 3, 10, 30, 100, 300, and 1000 μ M) were measured. The maximum active tensions developed were recorded for each concentration. The highest active tension developed for each dose of the used stimulant was considered as the maximal response to the stimulant. After the experiment, the segments were air-dried and weighed.

The responses were expressed as a gram force/gram tissue (g/g). The frequency of EFS (EFS-EC₅₀) and the

cumulative dose of ACh (ACh-EC₅₀) causing 50% of the maximal responses were calculated and represented as the sensitivity of airway smooth muscle.

Determination of IL-4 and IFN- γ in BAL Fluid

Cell-free BAL fluids were stored at -20°C until analysis of cytokines. The quantities of IL-4 and IFN- γ in the supernatant of BAL fluid were determined by using commercially available enzyme-linked immunosorbent assay (ELISA) kits (BioSource Cytoscreen, California). The standard curves were generated by the standards of known IL-4 or IFN- γ content provided. Sensitivities were 2 pg/ml for IL-4 and 13 pg/ml for IFN- γ .

Statistical Analysis

The mean values of response obtained from each tracheal ring segment in each rat were used in statistical analyses. Results were expressed as means $\pm$ SEM values. Mann-Whitney *U* test was used to determine the level of difference between two groups. A *P* value of less than 0.05 was considered significant.

RESULTS

BCG Infection Suppresses Airway Muscle Sensitivity and Airway Eosinophilia

The sensitivity of tracheal smooth muscle to EFS was significantly lower in rats infected with BCG after OVA sensitization than in the uninfected control rats (*P* < 0.001) (Fig. 2A). Maximal isometric contraction of tracheal smooth muscle to EFS in rats infected after sensitization (169.88 $\pm$ 14.37 g/g) was slightly lower than that in controls (218.80 $\pm$ 23.01 g/g), without a statistical significance. Similarly, the sensitivity of the smooth muscle to ACh was significantly lower in rats infected with BCG after sensitization than in the control rats (*P* < 0.01) (Fig. 2B). Maximal isometric contraction of the smooth muscle to ACh in rats infected after sensitization (356.19 $\pm$ 27.67 g/g) was slightly lower than that in controls (428.34 $\pm$ 39.10 g/g), without a significance. In an additional experiment, airway smooth muscle responsiveness was measured in five nonsensitized and nonprovoked normal male SD rats. Their body weight ranged from 270 to 295 g. The smooth muscle sensitivities to EFS and ACh were 14.07 $\pm$ 0.94 Hz and 27.07 $\pm$ 4.23 μ M, respectively. The maximal isometric contractions to EFS and ACh were 187.14 $\pm$ 23.73 and 366.71 $\pm$ 37.52 g/g, respectively. The sensitivities to EFS and ACh in OVA-sensitized and OVA-provoked rats were

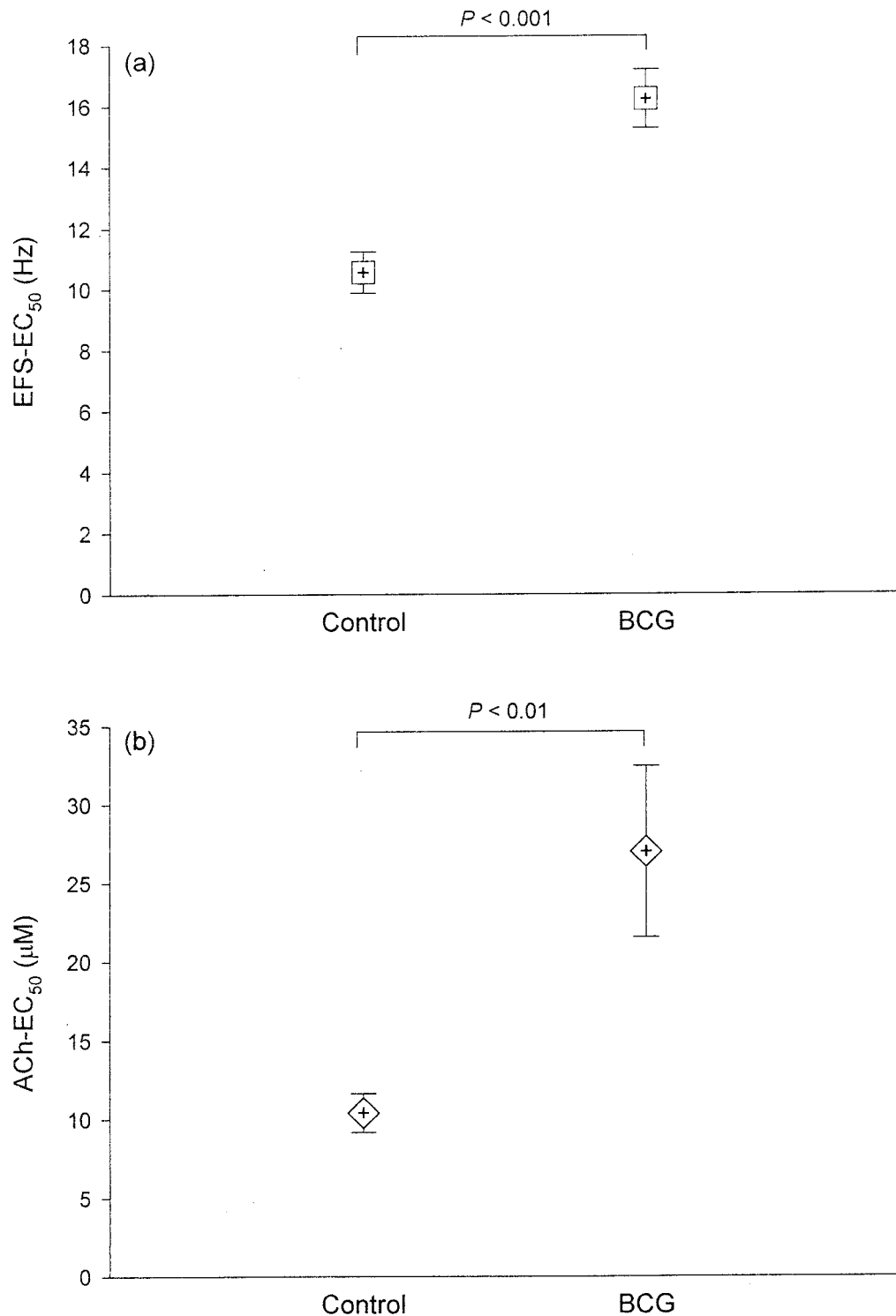


Fig. 2. The effects of bacille Calmette–Guérin (BCG) infection after ovalbumin (OVA) sensitization on the sensitivity to (a) electrical field stimulation (EFS) and (b) acetylcholine (ACh). Male Sprague-Dawley rats were sensitized with 10 μg OVA and injected with saline alone (control group, $n = 9$) or 6×10^5 colony-forming units (CFUs) of BCG (BCG group, $n = 9$) 11 days after OVA sensitization. Three days after BCG inoculation, the rats were provoked with 5% OVA. Isometric tension of tracheal smooth muscle was measured 24 hours after OVA provocation. Values are presented as means $\pm$ SEM.

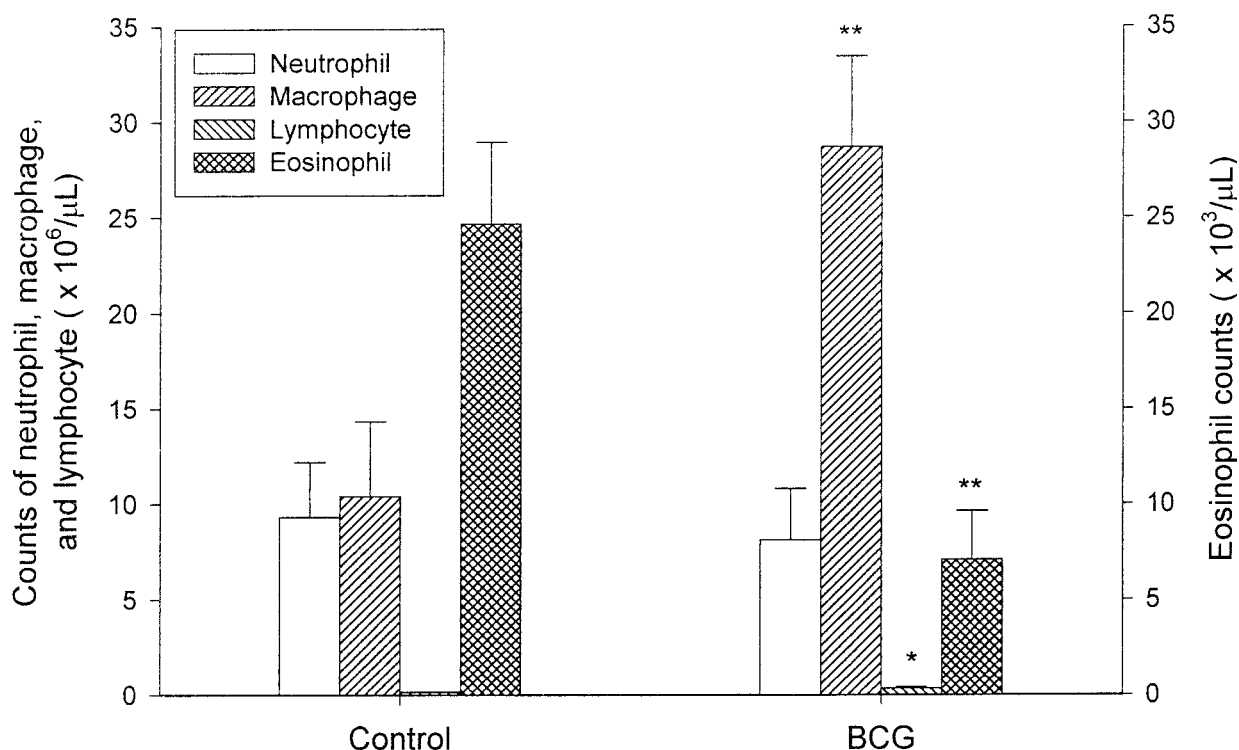


Fig. 3. The effect of bacille Calmette–Guérin (BCG) infection after ovalbumin (OVA) sensitization on airway inflammatory cells, such as neutrophils and macrophages, lymphocytes, and eosinophils. Rats were injected with saline alone (control group, $n = 9$) or BCG (BCG group, $n = 9$) after OVA sensitization, as described in the legend to Fig. 1. Bronchoalveolar lavage (BAL) was done 24 h after OVA provocation. Values are expressed as means $\pm$ SEM; * and ** represent $P < 0.05$ and $P < 0.01$ compared with controls, respectively.

significantly increased as compared with nonsensitized and nonprovoked normal rats ($P < 0.01$). The sensitivities to EFS and ACh in BCG infected OVA-sensitized and OVA-provoked rats did not differ from those in nonsensitized and nonprovoked normal rats ($P > 0.05$). These results suggest that the sensitivities to EFS and ACh are significantly increased by OVA sensitization and provocation and the increased sensitivity is normalized by BCG infection.

There was no significant difference of the absolute counts of neutrophils in BAL fluid between BCG-infected rats and controls. The counts of macrophages and lymphocytes were significantly increased in BAL fluid in rats with infection with BCG compared with controls ($P < 0.01$ and, $P < 0.05$, respectively). However, eosinophil counts in BAL fluid were significantly decreased in rats infected with BCG compared with the uninfected control rats ($P < 0.01$) (Fig. 3). These data suggest that BCG infection in allergen-presensitized rats may modulate eosinophilic airway inflammation induced by the allergen.

BCG Infection Inhibits Th2 Immune Response

The quantities of IL-4 in BAL fluid significantly decreased in BCG-infected rats compared with the uninfected control rats ($P < 0.01$). The amount of IFN- γ in BAL fluid was slightly higher in rats with BCG infection than in controls, without a statistical significance (Table I). Largely as a result of the decreased IL-4 production, the ratio of IL-4–IFN- γ was significantly lower in BCG-infected rats than in controls ($P < 0.01$); (Fig. 4). In an additional experiment, the levels of IL-4 and IFN- γ were measured in five nonsensitized and nonprovoked normal male SD rats and five only BCG-infected male SD rats. Their body weight ranged from 270 to 300 g. The BCG-infected rats were infected intraperitoneally

Table I. Cytokine Production in BAL Fluids

	Control ($n = 9$)	BCG ($n = 9$)	P value
IL-4 (pg/ml)	27.41 $\pm$ 6.02	3.43 $\pm$ 1.45	<0.01
IFN- γ (pg/ml)	403.15 $\pm$ 83.08	475.34 $\pm$ 67.37	>0.05

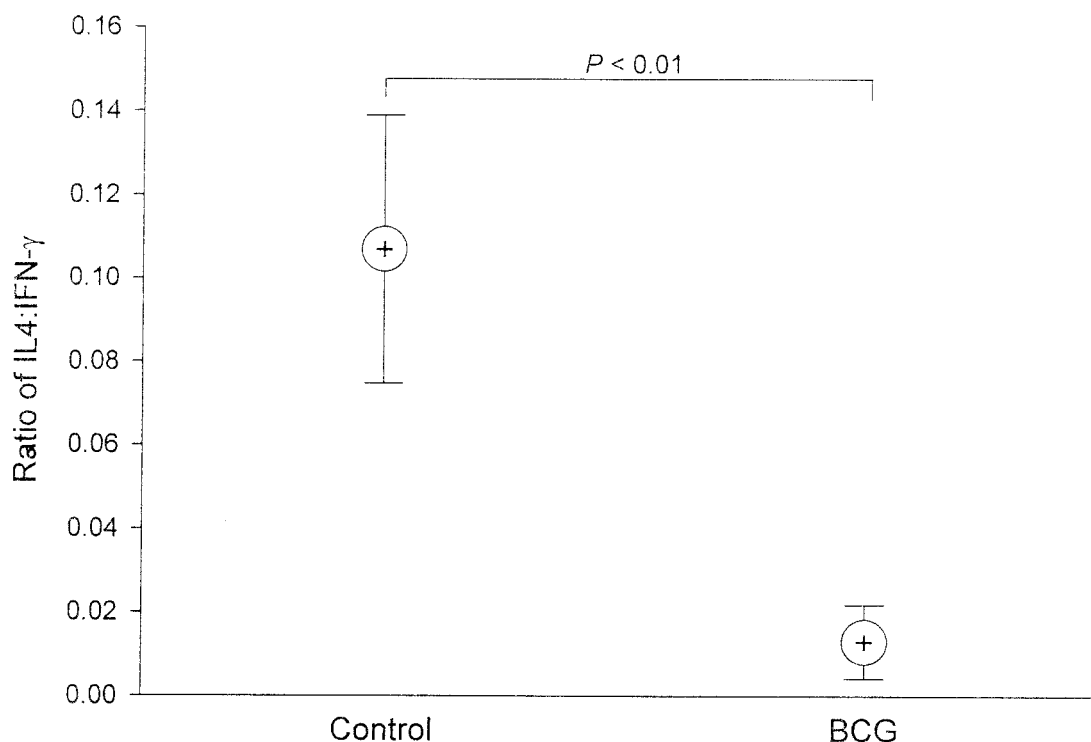


Fig. 4. The effect of bacille Calmette–Guérin (BCG) infection after ovalbumin (OVA) sensitization on Th2 immune response. Rats were injected with saline alone (control group, $n = 9$) or BCG (BCG group, $n = 9$) after OVA sensitization, as described in the legend to Fig. 1. Bronchoalveolar lavage (BAL) was done 24 h after OVA provocation. IL-4 and IFN- γ in BAL fluid were measured using a sandwich ELISA. Ratio of IL-4–IFN- γ was calculated. Values are expressed as means $\pm$ SEM.

with 6×10^5 CFUs of live attenuated BCG 4 days before analysis of cytokines. The levels of IL-4 in both nonsensitized and nonprovoked normal rats and only BCG-infected rats were ≤ 2 pg/ml. The level of IFN- γ was significantly higher in only BCG-infected rats (600.2 ± 74.8 pg/ml) than in normal rats (353.6 ± 60.45 pg/ml; $P < 0.05$), suggesting that BCG infection induces a Th1 immune response. These results indicate that BCG infection in OVA-presensitized rats can modulate preestablished Th2 immune response.

DISCUSSION

The idea that the cytokine profiles of resting memory T-cell populations are relatively flexible and can be reprogrammed is an important concept (17), which has significant implications for therapy of allergic diseases, in particular, bronchial asthma. Because BCG has been shown to be very potent inducer of the Th1 immune response (18), the present study addressed the question of whether BCG infection in OVA-presensitized rats can suppress Th2 immune responses and allergic asthmatic reactions to OVA exposure. A major finding of this study

was that BCG infection after sensitization with OVA inhibited airway muscle sensitivity, eosinophilic inflammation, and Th2 cytokine production induced by OVA sensitization and provocation. Recently, Wang and Rook (16) showed an injection of *M. vaccae* into OVA-preimmunized mice suppressed Th2 immune responses. Our data extended their findings by showing that mycobacterial infection in allergen-presensitized rats inhibits the development of AHR and eosinophilic airway inflammation, which are characteristics of bronchial asthma.

AHR in this study was represented as the sensitivity of airway smooth muscle to cholinergic stimuli, such as EFS and ACh. Although the exact pathogenic mechanisms of AHR are unknown, intrinsic physiological properties of the smooth muscle itself may be suggested (19). In our previous study, the sensitivity of airway smooth muscle to cholinergic stimuli significantly increased in OVA-sensitized and OVA-provoked rats compared with nonsensitized and nonprovoked control (20). Some studies also have reported that the sensitivity of airway smooth muscle increases in experimental asthmatic model (21, 22). Herz *et al.* (13) also assessed AHR

by the response of tracheal smooth muscle to EFS and reported that BCG immunization before OVA sensitization prevented the development of AHR. In the present study, BCG infection after OVA sensitization significantly suppressed the development of AHR. In addition, the suppression of increased sensitivity of airway smooth muscle in BCG-infected rats was accompanied with the reduction of airway eosinophilia. Although Erb *et al.* (23) demonstrated that intranasal BCG infection reduced allergen-induced airway eosinophilia, the relevance of this reduction to a clinical asthmatic reaction is not clear because an asthmatic reaction can be induced by various allergens in the absence of airway eosinophilic inflammation (24). Our results, however, showed the suppression of both airway eosinophilia and AHR, which are important components of the asthmatic reaction, thus indicating the inhibitory effect of BCG infection after allergen sensitization on allergic asthmatic reaction.

Our study demonstrated an IL-4 production and a ratio of IL-4-IFN- γ production significantly decreased in BCG-infected rats. This suggests that BCG infection in presensitized rats decreases Th2 immune responses. It is tempting to speculate that the balance between IL-4 and IFN- γ present at the site of T-cell activation determines whether Th1 or Th2 cell is generated, with relatively low IL-4 levels being susceptible to the inhibitory effect of IFN- γ on Th2 development. It has been suggested that the ratio of Th1-Th2 cytokine production elicited by allergen exposure was critical in determining the type of immune responses (25).

How can BCG infection in sensitized rats with preestablished Th2 memory modulate cytokine-producing patterns and inhibit allergic asthmatic reaction induced by allergen? IL-4 down-modulates the expression of the IL-12R β chain on T cells *in vitro*. This down-modulation leads to the generation of Th2 cells, since they are no longer responsive to IL-12-mediated signaling (26). However, the presence of IFN- γ may inhibit this IL-4-induced effect. Therefore, allergen-specific Th2 memory cells may produce Th1 cytokines when activated in the presence of IL-12, a potent IFN- γ -inducing protein (27), before the more malleable resting memory T cells differentiate into end-stage effector cells (28) in which Th2 effector cells lose IL-12 receptor function (29).

The advance of molecular methodologies opens up other promising new prophylactic or therapeutic approaches for immunomodulation. These include direct mucosal Th1 cytokine gene transfer approaches and manipulation of the immune system by using DNA-based immunization. Li *et al.* (30) transferred IFN- γ genes in a plasmid expression vector into mouse lung

mucosa, using a murine model for airway eosinophilia and hyperreactivity. They showed a time-dependent increase in IFN- γ levels in BAL fluid and a significant decrease in the levels of airway hyperreactivity. As a corollary, Hogan *et al.* (31) showed that transfer of the IL-12 gene into the lung inhibited local Th2 cytokine production and suppressed the development of airway eosinophilia and hyperreactivity. It was discovered that short palindromic DNA segments containing CpG motifs within the sequences of commonly used plasmid vectors reside and these motifs were found to be potent inducers of Th1 responses (32). In fact, when CpG-containing oligonucleotides, together with immunizing antigen, were tested in a mouse model for asthma, allergen-induced inflammation could be prevented (33). However, despite these successes, the molecular basis remains unclear, and the regulatory T-cell populations responsible for mediating suppression remain controversial. Thus further understanding of regulatory mechanisms is needed to determine potential clinical utility.

The efficacy of BCG infection in rats may be different from that of the extrapulmonary BCG vaccination in human. There are now reported retrospective analyses of BCG vaccination programs in relation to atopy, but their findings are conflicting. Several studies have shown no association between BCG vaccination and development of atopy and/or asthma symptoms later in life (34, 35), while Aaby *et al.* (36) reported less atopy (by allergen skin prick testing) in infants receiving BCG in Guinea-Bissau, particularly if the BCG was administered in the first week of life. It is very difficult to compare these studies and to draw firm conclusions from them, particularly deciding whether or not BCG given by intradermal injection inhibits atopy in humans or whether it might if given very early in life. Therefore, prospective controlled studies are needed to test the potential of mycobacterial preparations or products given in early life to limit atopic disorder in humans. BCG vaccination by intradermal inoculation represents one safe and tested method for investigation, but the advance of molecular methodologies may open up the potential of a live recombinant BCG (rBCG) allergy vaccine. Kumar *et al.* (37) have shown that rBCG-secreting antigens or allergens may be utilized for the induction of a Th1-like response and the down-regulation of IgE antibody response.

The limitation of our study was that OVA nonsensitized and nonprovoked control group and only BCG-infected group were not included in this study. It may not be clear whether OVA sensitization and provocation develops Th2 immune response and allergic asthmatic reaction in our rats. However, we demonstrated in our previous study that sensitization and provocation *in vivo*

with OVA in SD rats significantly increased the sensitivity of airway smooth muscle to cholinergic stimuli compared with nonsensitized and nonprovoked control. The sensitivity correlated well with airway eosinophilia and was highest when sensitized with 10 μ g OVA and exposed to 5% OVA aerosols (20). The same sensitizing and provoking doses of OVA were used in the present study. It is considered that OVA sensitization and provocation in those rats induce allergic asthmatic reaction. Furthermore, our additional experiments shown in the results indicated that OVA sensitization and provocation increased the airway smooth muscle sensitivity and Th2 immune response. We also demonstrated that BCG infection in normal rats induced Th1 immune response.

Unlike other studies (13–15), BCG was infected after allergen sensitization in our study. The BCG infection in presensitized rats significantly inhibits Th2 immune response and asthmatic reaction to subsequent allergen exposure. The findings may support the hypothesis that childhood infections suppress allergic disease. It is possible from these results that BCG vaccine may be used as immunomodulating agent to switch off atopy for the presensitized host with Th2 memory.

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