

Airway Hyperresponsiveness, Late Allergic Response, and Eosinophilia Are Reversed with Mycobacterial Antigens in Ovalbumin-Presensitized Mice¹

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Pretreatment with mycobacterial Ags has been shown to be effective in preventing allergic airway inflammation from occurring in a mouse model. Because most asthmatics are treated after the development of asthma, it is crucial to determine whether mycobacterial Ags can reverse established allergic airway inflammation in the presensitized state. Our hypothesis, based upon our previous findings, is that mycobacteria treatment in presensitized mice will suppress the allergic airway inflammation with associated clinical correlates of established asthma, with the noted exception of factors associated with the early allergic response (EAR). BALB/c mice sensitized and challenged with OVA were evaluated for pulmonary functions during both the EAR and late allergic response, and airway hyperresponsiveness to methacholine. Following this, sensitized mice were randomized and treated with placebo or a single dose (1×10^5 CFUs) of bacillus Calmette-Guérin (BCG) or *Mycobacterium vaccae* via nasal or peritoneal injection. One week later, the mice were rechallenged with OVA and methacholine, followed by bronchoalveolar lavage (BAL) and tissue collection. Mice treated with intranasal BCG were most significantly protected from the late allergic response ($p < 0.02$), airway hypersensitivity ($p < 0.001$) and hyperreactivity ($p < 0.05$) to methacholine, BAL ($p < 0.05$) and peribronchial ($p < 0.01$) eosinophilia, and BAL fluid IL-5 levels ($p < 0.01$) as compared with vehicle-treated, sensitized controls. Intranasal *M. vaccae* treatment was less effective, suppressing airway hypersensitivity ($p < 0.01$) and BAL eosinophilia ($p < 0.05$). No changes were observed in the EAR, BAL fluid IL-4 levels, or serum total and Ag-specific IgE. These data suggest that mycobacterial Ags (BCG $\gg$ *M. vaccae*) are effective in attenuating allergic airway inflammation and associated changes in pulmonary functions in an allergen-presensitized state. *The Journal of Immunology*, 2002, 168: 2516–2522.

The characterization of type 1 and type 2 cytokine responses from CD4⁺ T cells led to the hypothesis that Th1-stimulating Ags, such as the bacillus Calmette-Guérin (BCG)³ vaccine, would suppress the atopic Th2 response (1–3). Several experimental studies involving mycobacterial Ags in mouse models of allergic airway inflammation have been reported (4–9). These studies, while largely positive in their conclusion, share a common theme that may, at least in part, explain their potent antiasthmatic response. In these experiments (with one exception; Ref. 7), mice have been treated with mycobacteria either entirely before or during the allergen sensitization protocol (4–6, 8, 9). Mixed results from retrospective clinical data have been reported (10–13). These studies often included subjects immunized with BCG well into their first years of life and likely after allergen sensitization occurred. Therefore, we designed a study to reflect this possibly more likely temporal relationship between allergen sensitization and BCG vaccine exposure in a mouse model.

Rather unambiguous suppression of preexisting IgE responses in OVA-sensitized mice, as alluded to above, has been reported (7). However, previously published work by others and us (14) have suggested that mycobacterial Ags may not require down-regulation of IgE production to achieve suppression of airway eosinophilia and airway hyperresponsiveness (AHR) (5). These observations have called into question (in the mouse model) the absolute necessity of IgE suppression to attenuate the underlying pathology in asthma, that of airway narrowing. *Mycobacterium vaccae*, a nonpathogenic mycobacterial species, was specifically included in these experiments to test, within the same animals, its effects upon IgE and pulmonary function.

In this study, we investigated the ability of mycobacteria to suppress preexisting AHR and airway resistance as measured by enhanced pause (P_{enh}) associated with the early allergic response (EAR) and late allergic response (LAR). We hypothesized that treatment with mycobacterial Ags in Ag-presensitized and -challenged mice would reverse and suppress existing allergic airway inflammation and associated clinical correlates of established asthma in mice, excluding factors associated with the EAR.

We observed, for the first time, that previously existing AHR and airway resistance during the LAR in sensitized mice can be reversed with BCG treatment and, to a lesser extent, with *M. vaccae* treatment.

Materials and Methods

Animals

Four- to 5-wk-old female BALB/c mice were obtained from Harlan Laboratories (Indianapolis, IN) and housed according to the National Institutes of Health guidelines. The research protocol of this study was approved by the Animal Research Committee of Creighton University (Omaha, NE).

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³ Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; EAR, early allergic response; LAR, late allergic response; P_{enh} , enhanced pause; i.n., intranasal(ly); AUC, area under the curve.

Mice were housed in separate cages according to treatment. Food and water were provided ad libitum.

Mycobacteria preparation

Lyophilized BCG (Tice; Organon, West Orange, NJ) and *M. vaccae* (no. 29678; American Type Culture Collection, Manassas, VA) were cultured in Lowenstein-Jensen medium (REMEI, Lenexa, KS) at the Creighton University Pathology Lab. Three to 4 wk after the cultures were begun, the vials were centrifuged and resuspended in PBS. Logarithmic dilutions of the cells into sterile vials were made for both bacteria. Agar plates (7-H10; REMEI) corresponding to logarithmic dilutions were incubated for an additional 2 wk. The stock vials were frozen at -80°C . CFUs on the culture plates were counted and an estimation of the number of viable organisms in the stock vials was then made. Aliquots of 1×10^5 organisms ($50 \mu\text{l}$) in medium were made and refrozen at -80°C until needed.

Sensitization

Mice were sensitized on days 0 and 14 with an i.p. injection of $20 \mu\text{g}$ grade V chicken egg OVA (Sigma-Aldrich, St. Louis, MO) and 2 mg alum (Imject Alum; Pierce, Rockford, IL) suspended in PBS to a total volume of $100 \mu\text{l}$. This was followed by a daily administration of nebulized 1% OVA for 20 min from day 28 through day 30. Nonsensitized control animals received only the PBS (Fig. 1).

Noninvasive method for measuring pulmonary function

Single-chamber whole-body plethysmographs (Buxco Electronics, Troy, NY), without the use of anesthesia or restraint, were used to measure pulmonary functions. This method has been demonstrated to accurately reflect airway resistance (14, 15), expressed as the P_{enh} units (16).

Initial allergen challenge

All mice were placed in individual plethysmograph chambers on day 32 and baseline P_{enh} readings were taken. Subsequently, mice were challenged to 5% aerosolized OVA for 20 min. This was followed by recording of pulmonary functions during the EAR (0–30 min) and the LAR (1–7 h). The results of Ag challenges are expressed as the area under the curve (AUC), which takes into account the complete breadth of either the EAR or LAR. An individual mouse's baseline P_{enh} value served as the reference to which subsequent increases in P_{enh} were compared.

Initial methacholine challenge

Mice were challenged on day 33, 24 h post-OVA challenge, with increasing doses of aerosolized methacholine, and pulmonary functions were recorded using the Buxco whole-body plethysmograph system. An aerosol challenge at each dose was administered via an Ultra Neb-90 (DeVilbiss, Somers, PA) with the highest setting for exactly 1 min. A 1-min wash-out period followed. Immediately thereafter, data were recorded for 5 min and a mean of this time period, in terms of P_{enh} , was made. After the recording period, the P_{enh} values for each mouse were allowed to return to baseline before the next higher dose of methacholine was administered.

The results of methacholine challenges were transformed into the P_{enh} index, where increasing P_{enh} units for a given mouse are expressed in terms

of the fold increase from the baseline P_{enh} unit. Data from the methacholine challenges were compared in two different ways. First, the PC_{200} , the dose of methacholine at which a 200% increase in P_{enh} units was observed, was calculated for each animal and compared as a measure of airway hyper-sensitivity. Second, the maximum P_{enh} index for each animal was also compared as a measure of airway hyperreactivity.

Randomization and treatment

Following methacholine challenge, nonsensitized mice were equally and randomly divided into two groups (intranasal (i.n.) or i.p. treatment with vehicle) and sensitized mice were equally and randomly divided into six groups (i.n. or i.p. treatment with either vehicle, BCG or *M. vaccae*) and treated with the appropriate mycobacterial Ag (Fig. 1). Mice in i.n. experimental groups were anesthetized with ketamine and xylazine (20:1) followed by immunization with $50 \mu\text{l}$ of 1×10^5 CFUs of the appropriate organism. Mice in i.p. experimental groups were injected with $50 \mu\text{l}$ of 1×10^5 CFUs of the appropriate organism. Nonsensitized and sensitized control mice were treated only with the vehicle (PBS) via the appropriate route.

Final Ag and methacholine challenges

On day 40, Ag challenge was conducted exactly as previously described. A final methacholine challenge (as described) was administered on day 41.

BAL collection

Immediately following the final methacholine challenge the mice were euthanized with a lethal dose of pentobarbital. Tracheas were cannulated and lungs were washed with 1 ml PBS. Cytospin slides were prepared from each sample following lavage cell counting using a Coulter counter (Beckman Coulter, Fullerton, CA). Slides were stained with DiffQuik (Baxter Healthcare, McGaw Park, IL) for analysis of differential cell populations using standard morphological criteria.

Serum Ig analysis

Blood collected after sacrifice on day 41 was immediately centrifuged and serum was collected and stored at -70°C for later analysis. ELISA for both total and Ag-specific IgE was conducted as previously described (17) and according to the manufacturer's recommendations using rat anti-mouse IgE (BD Pharmingen, San Diego, CA), standard IgE (BD Pharmingen), and rat anti-mouse IgE-HRP (Southern Biotechnology Associates, Birmingham, AL) for the total IgE assay, with the substitution of biotinylated OVA (Immunoprobe biotinylation kit; Sigma-Aldrich), followed by addition of streptavidin-HRP (BD Pharmingen), for the Ag-specific assay. Both cytokine and Ig assays were developed with 3,3',5,5'-tetramethylbenzidine substrate and read at 450 nm using a Bio-Rad microplate reader and software (Bio-Rad, Hercules, CA). Sensitivity for total IgE was 1 ng/ml. Ag-specific IgE results are expressed in units of absorbance (OD).

Cytokine analysis

Cytokines were measured in the supernatants of bronchoalveolar lavage (BAL) fluid and/or serum. Ab pairs and protein standards for IL-4, IL-5, and IFN- γ (BD Pharmingen), as well as IL-3 and TGF- β_1 (R&D Systems, Minneapolis, MN) were used according to the manufacturer's recommendations. Sensitivities for the assays were 12, 8, 9, 8, and 5 pg/ml, respectively.

Histology

Whole lungs were removed, set in tissue freezing medium (Triangle Bio-medical Sciences, Durham, NC), and frozen immediately in liquid nitrogen. Sections of $8\text{-}\mu\text{m}$ thickness were prepared and stained with H&E. Slides were analyzed under low power ($\times 10$) for a determination of total peribronchial inflammation. Semiquantitative analysis was achieved by assigning a value of 0 for no inflammation, 1 for mild inflammation, 2 for moderate inflammation, and 3 for severe inflammation (see Fig. 2). Under higher power magnification ($\times 40$), eosinophilic infiltration was determined by counting the number of eosinophils within the inflamed peribronchial region and expressing this as a percentage.

Statistical analysis

Data were analyzed using GraphPad PRISM statistical analysis and graphing software (GraphPad, San Diego, CA). For pulmonary function assays, the analysis of variance using all treatment groups was used to determine

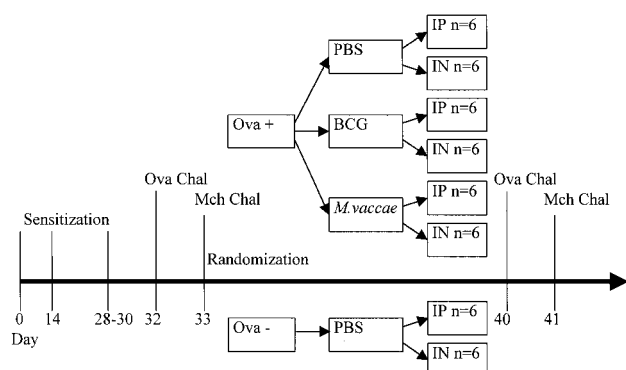


FIGURE 1. Schematic representation of experiment protocol. Mice were sensitized to OVA as described in *Materials and Methods*. Randomization and treatment (see *Materials and Methods*) were started immediately following methacholine challenge on day 33.

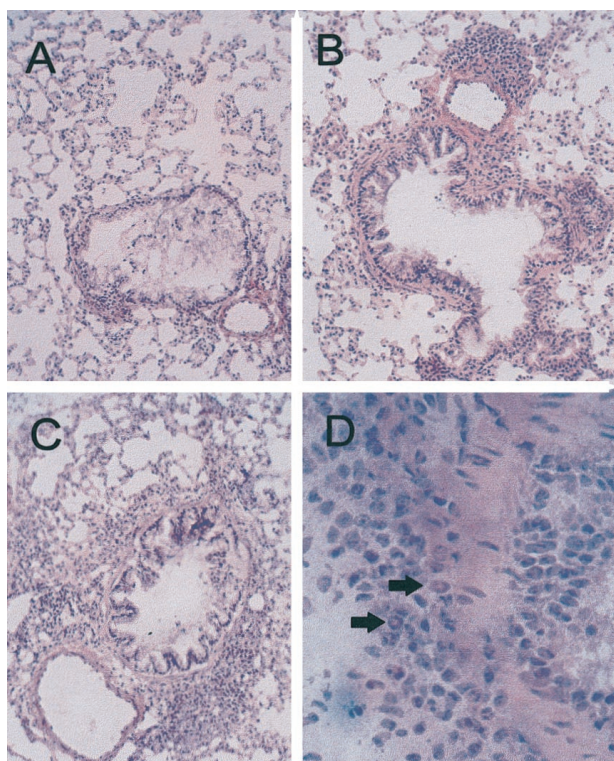


FIGURE 2. Representative H&E-stained murine lung sections. Sections were graded for peribronchial inflammation and scaled separately for percentage of peribronchial eosinophilia. Shown are representative sections for grade 0 (no inflammation, $\times 10$) (A); grade 1 (mild inflammation, $\times 10$) (B); grade 2 (moderate inflammation, $\times 10$) (C); and peribronchial eosinophils (arrows, $\times 40$) (D).

differences between both the experimental or control groups and the pre-randomized sensitized animals (on day 40/41 vs day 32/33), as well as between nonsensitized and sensitized control groups (on day 40/41). All other assays were also compared using analysis of variance. Values given are means $\pm$ SEM from at least six animals in each group unless otherwise noted. A value of $p < 0.05$ was considered significant.

Results

i.n. BCG administration suppresses specific airway resistance during the LAR in presensitized animals

One week following mycobacterial Ag inoculation to presensitized mice, the animals were again challenged with aerosolized 5% OVA and monitored for changes in airway resistance as described. There was a significant increase in the EAR and LAR in OVA-sensitized and -challenged group (Figs. 3 and 4). None of the treatment groups exhibited a significant change in the EAR (Fig. 4A). Only those animals that received *i.n.* BCG showed a significantly suppressed response in the LAR (Fig. 4B). Statistical comparison between the nonsensitized and sensitized control mice for the airway resistance during the EAR and LAR on both days 32 and 40 showed significant differences in the EAR ($p < 0.001$) as well as the LAR ($p < 0.001$), while no significant changes after the 8 days were seen within each control group.

*Mycobacterial administration suppresses airway hypersensitivity, while only *i.n.* BCG treatment suppressed both airway hypersensitivity and airway hyperreactivity in presensitized animals*

There was a significant increase in both airway hypersensitivity and airway hyperreactivity in OVA-sensitized and -challenged mice (Fig. 5). The PC_{200} values to methacholine were used as a

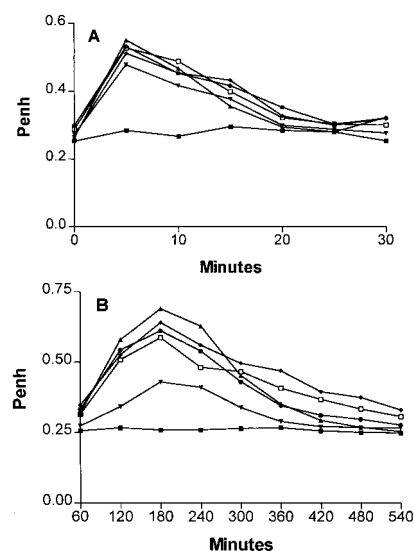


FIGURE 3. EAR (A) and LAR (B) following challenge with OVA on day 40. P_{enh} values were recorded in individual animals. The data are representative of six animals in each experimental group: nonsensitized control (■), OVA-sensitized and challenged (▲), *i.n.* BCG (▼), *i.p.* BCG (◆), *i.n.* *M. vaccae* (●), and *i.p.* *M. vaccae* (□).

measure of airway hypersensitivity in this experiment, as mice typically reach this point early in the dose response curve. Highly significant suppression was observed for each experimental group (Fig. 5B). As a measure of airway hyperreactivity, the maximum P_{enh} index was used, which reflected the highest degree of airway resistance as measured by this assay. Only the animals treated *i.n.* with BCG showed significant reduction in maximum P_{enh} index (Fig. 5C). Differences between nonsensitized and sensitized control mice were significant for both parameters on either of day 32 and day 40 ($p < 0.01$), while no significant changes were observed after 8 days within each control group.

Reductions in specific BAL leukocyte populations were split among various treatment groups

Lavage fluid was collected following methacholine challenge on day 41. Eosinophils were significantly reduced only in the *i.n.* experimental groups, to statistically the same degree in each (Table I). Lymphocytes, the only other group of white cells to be attenuated in the experiment, were reduced only in the *M. vaccae*-treated animals, regardless of Ag delivery route. Finally, while both *i.n.* treatment groups yielded apparent reductions in total BAL leukocytes, only the *M. vaccae*-treated animals showed a significant reduction.

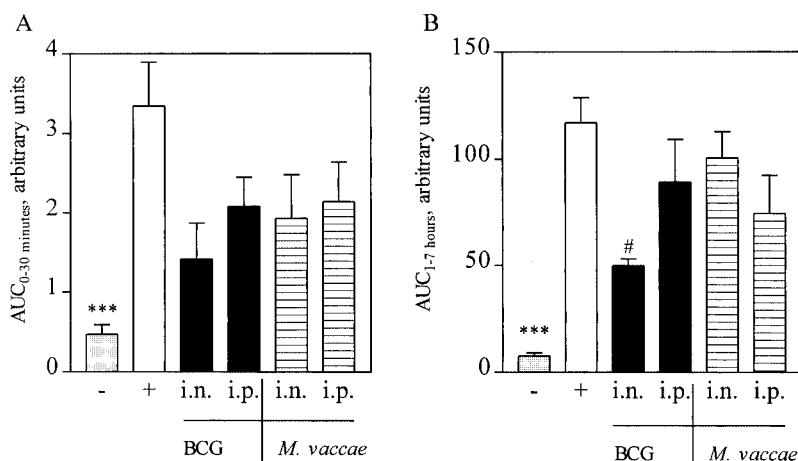
Neither total serum IgE nor OVA-specific IgE is suppressed after mycobacteria treatment

While very significant increases were seen in sensitized control animals compared with nonsensitized animals, no reductions compared with sensitized animals were seen with any experimental group for either total IgE or OVA-specific IgE (Table II).

*BAL fluid IL-5 concentrations are suppressed with mycobacterial treatment, while only *i.n.* BCG treatment increased BAL fluid IFN- γ levels*

Supernatants collected from BAL fluid were analyzed for the presence of several cytokines. IL-5 levels were significantly reduced in all treatment groups, except for *i.n.* *M. vaccae* (Table III). Interestingly, IFN- γ was only detected in the BAL fluid from mice

FIGURE 4. Effect of mycobacterial Ags on EAR and LAR in presensitized mice. Before randomization and treatment, all mice were challenged with OVA and pulmonary function was recorded during both the EAR (A, open bar) and LAR (B, open bar). One week following mycobacterial treatment, mice were rechallenged with OVA in an identical fashion. A, No suppression of the airway resistance, as measured by the AUC, during the EAR (0–30 min after OVA challenge) was observed in any treatment group. B, Airway resistance, as measured by the AUC, during the LAR (1–7 h after OVA challenge) was suppressed only with i.n. BCG treatment. Significant differences were observed for the EAR (***, $p < 0.001$) and the LAR (***, $p < 0.001$) between sensitized and nonsensitized controls. $n = 6$ for all groups; #, $p < 0.02$ as compared with the OVA-sensitized and -challenged group (+).



treated with i.n. BCG. IL-4 levels, while tending toward lesser amounts in all treatment groups, were not found to be significantly decreased after mycobacterial Ag exposure. TGF- β was observed at insignificantly increased levels in almost all experimental groups compared with sensitized controls. IL-3 levels could not be detected in our BAL assays. There was no significant difference in serum IFN- γ levels when compared between the treatment and sensitized control groups. Assays for serum IL-3, IL-4, and IL-5 were, unfortunately, not sufficiently sensitive.

Eosinophilic infiltration of the peribronchial area is significantly suppressed with BCG treatment

Lung sections were inspected for both an overall semiquantitative score of inflammation as well as a measure of the degree of eosinophilic infiltration. Postsensitization treatment with mycobacterial Ags did not have any effect on the degree of peribronchial

inflammation (Figs. 1 and 6A). However, the relative number of eosinophils infiltrating the peribronchial region was significantly reduced only with BCG treatment of either route, while apparent, yet insignificant, reductions were also seen with *M. vaccae* treatment (Figs. 1 and 6B).

Discussion

This study examined the effects of two different mycobacterial Ags and two different routes of administration on OVA-presensitized mice. A comprehensive follow-up of numerous allergic parameters was incorporated in an attempt to direct the formation of hypothesis accounting for the observed effects. While evidence suggests that direct, local delivery of mycobacterial Ags is superior to systemic delivery in suppressing allergic responses in the murine model (5), the fact that both work to varying degrees may be useful in determining mechanisms.

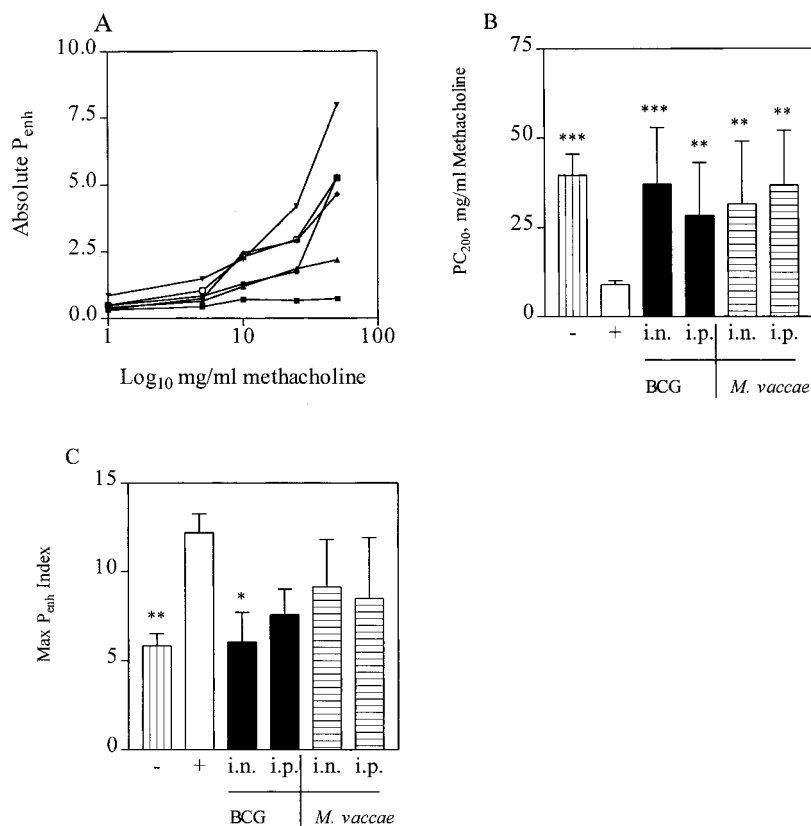


FIGURE 5. Effect of mycobacterial Ags on airway hypersensitivity and hyperreactivity to methacholine in presensitized mice. Before randomization and treatment and 24 h following OVA challenge, mice were challenged with increasing concentrations of aerosolized methacholine. A, One week following mycobacterial treatment and 24 h following OVA challenge, the methacholine challenge was repeated and a dose response curve was constructed in each experimental group: nonsensitized control (■), OVA-sensitized and challenged (▲), i.n. BCG (▼), i.p. BCG (◆), i.n. *M. vaccae* (●), and i.p. *M. vaccae* (□). B, PC₂₀₀ values were determined and presented as a measure of airway hypersensitivity. All mice treated with mycobacteria showed very significant suppression, with the greatest effect in the BCG i.n. group. C, The maximum P_{enh} index refers to the greatest observed fold increase over baseline P_{enh} values and is presented as a measure of airway hyperreactivity. Only mice treated with BCG i.n. showed significant suppression for this parameter. Statistically significant values were observed for both PC₂₀₀ values ($p < 0.001$) and maximum P_{enh} index values ($p < 0.01$) between sensitized and nonsensitized controls. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ as compared with the OVA-sensitized and -challenged group (+).

Table I. Effect of mycobacterial Ags on BAL cells (raw values $\times 10^3$)

Sensitized	Treatment	Total Cells	Mac	Neu	Lym	Eos
—	Vehicle i.n.	50.3 $\pm$ 3 ^a	49 $\pm$ 3 ^b	1.3 $\pm$ 0.4 ^b	0.05 $\pm$ 0.05 ^a	0
—	Vehicle i.p.	51.2 $\pm$ 8 ^c	48 $\pm$ 7 ^b	2.3 $\pm$ 1.5 ^b	0.5 $\pm$ 0.3 ^a	0
+	Vehicle i.n.	450 $\pm$ 93	130 $\pm$ 28	29 $\pm$ 14	38 $\pm$ 11	263 $\pm$ 71
+	Vehicle i.p.	466 $\pm$ 42	100 $\pm$ 14	31 $\pm$ 13	52 $\pm$ 12	283 $\pm$ 42
+	BCG i.n.	317 $\pm$ 39	158 $\pm$ 22	35 $\pm$ 9	20 $\pm$ 5	104 $\pm$ 25 ^b
+	BCG i.p.	527 $\pm$ 63	215 $\pm$ 19	52 $\pm$ 18	28 $\pm$ 10	231 $\pm$ 46
+	<i>M. vaccae</i> i.n.	202 $\pm$ 51 ^b	99 $\pm$ 25	20 $\pm$ 8	9 $\pm$ 4 ^b	73 $\pm$ 21 ^b
+	<i>M. vaccae</i> i.p.	371 $\pm$ 42	151 $\pm$ 14	29 $\pm$ 10	8 $\pm$ 4 ^a	183 $\pm$ 32

^a Value of $p < 0.01$.^b Value of $p < 0.05$.^c Value of $p < 0.001$.

Intranasal BCG treatment (one dose, 1×10^5 CFUs) was the most effective combination in reversing Ag-induced asthma in this study. It was effective in reducing P_{enh} , which correlates well with airway resistance and intrapleural pressure (16) during the LAR (Fig. 4B), both airway hypersensitivity and hyperreactivity to methacholine (Fig. 5), BAL eosinophilia (Table I), BAL IL-5 (Table III), and peribronchial eosinophilia (Fig. 6). Other combinations, while variably effective in terms of cellularity and cytokines, did not differ among each other in terms of their effects on pulmonary functions. While these data cannot discern the exact mechanism, the observations of highly significant reductions in peribronchial eosinophilic infiltration and BAL fluid IL-5 concentrations in samples from animals treated i.n. with BCG do implicate the role of the eosinophil. Indeed, many reports have also positively correlated eosinophils and/or IL-5 with AHR (18–20). However, there have been negative reports as well, suggesting that eosinophils are neither required nor sufficient to induce AHR (21). In other reports, treatment with mAb to IL-5 had no effect on the reversal of established AHR in mice (22) and humans (23) despite complete suppression of eosinophil accumulation of airway tissue. Further studies, therefore, are clearly warranted to understand the role of eosinophils in AHR.

None of the treatments abrogated IgE (total or OVA-specific), IL-4, or OVA-induced airway resistance associated with the EAR. These findings are consistent with our previous observations that mycobacterial pretreatment also failed to prevent this set of parameters (14).

In addition to us, others have demonstrated a suppressive effect on various parameters of allergic inflammation in mouse models after mycobacteria pretreatment (4, 5, 8, 9). However, except for one report from the work of Wang and Rook (7), there is no information as to the potential for these Ags in attenuating a preexisting allergic state. This is not an insignificant detail, as envisioning future immunotherapies for allergic asthma must certainly take

into account presensitized individuals in addition to aiming to prevent sensitization altogether.

In light of the potential clinical application of the literature in this area, the stark contrast of our findings on the effect of mycobacterial Ags on serum IgE to those of Wang and Rook (7) becomes all the more important. Similarities between the study designs include very relevant factors of gender and genetic strain, while the differences may account for the discrepancies. Wang and Rook (7) found total IgE suppression (with neither OVA-specific IgE nor IgG1 being suppressed) with 10^7 – 10^9 CFUs of *M. vaccae*. The differences in our results from those of Wang and Rook (7) could be due to the differences in the type and amount of *M. vaccae* used. For example, they used attenuated *M. vaccae* and 100–10,000 times more Ag than our protocol. Erb et al. (5), using BCG treatment at the outset of OVA sensitization, found no change in either IgE or IgG1 with doses on the order of 10^5 CFUs, while other allergic parameters were nonetheless suppressed. We did not include such a high range of concentrations, as this lower dose has consistently suppressed the central parameters of AHR, airway resistance during the LAR, and eosinophilia (14, 24).

One of the hypotheses we have proposed as a result of our research is that mycobacterial Ags suppress asthma-like parameters independent of IgE. This is consistent with the reports of other investigators who have shown that IgE is not required for the development of eosinophilic airway inflammation and AHR in mice (21, 25–27). This could also be true in humans as observed by Haselden et al. (28). These investigators demonstrated that an intradermal injection of a linear peptide sequence within an allergen at a high dose can directly initiate a MHC-restricted, T cell-dependent late asthmatic reaction, without the requirement for an early IgE mast cell-dependent response in sensitized asthmatic subjects (28). These data support our model of airway inflammation and AHR. Furthermore, should this prove true in most of the asthmatic subjects, the entire breadth of the type 2 response, such as IL-4 and IgE, may not necessarily demand suppression, easing concerns of the notion of a pendulum swinging too far to the type 1 T cell response side (and unintended effects that may accompany this) and enabling novel approaches toward conventional immunotherapy. Indeed, the current therapy of choice for moderate to severe asthmatics is inhaled glucocorticoids, which themselves do little to suppress IgE levels (29–31).

Attempts to reverse or suppress a preexisting type 2 cytokine-weighted state raises questions of the stability and plasticity of Th cytokine secretion profiles. Ohta et al. (32) demonstrated a delayed-type hypersensitivity response in BALB/c mice adoptively transferred with Th1 cells primed in vitro, a response that lasted several months. Infants, too, are believed to possess a Th2-weighted Th repertoire (33), and recent evidence supports the hypothesis that in utero allergic sensitization occurs (34). Marchant et

Table II. Effect of mycobacterial Ags on total and OVA-specific serum IgE

Sensitized	Treatment	Total Serum IgE (ng/ml)	OVA-Specific IgE (OD)
—	Vehicle i.n.	78.6 $\pm$ 7 ^a	0.53 $\pm$ 0.03 ^b
—	Vehicle i.p.	23.4 $\pm$ 6 ^a	0.5 $\pm$ 0.02 ^b
+	Vehicle i.n.	271 $\pm$ 36	1.4 $\pm$ 0.2
+	Vehicle i.p.	337 $\pm$ 68	1.6 $\pm$ 0.1
+	BCG i.n.	290 $\pm$ 58	1.5 $\pm$ 0.1
+	BCG i.p.	313 $\pm$ 62	1.5 $\pm$ 0.1
+	<i>M. vaccae</i> i.n.	341 $\pm$ 76	1.5 $\pm$ 0.03
+	<i>M. vaccae</i> i.p.	296 $\pm$ 57	1.4 $\pm$ 0.1

^a Value of $p < 0.05$.^b Value of $p < 0.001$.

Table III. Effect of mycobacterial Ags on cytokines in presensitized mice^a

Sensitized	Treatment	BAL					Serum (IFN- γ)
		IL-5	IL-4	TGF- β	IFN- γ		
–	Vehicle i.n.	161 $\pm$ 29 ^b	73 $\pm$ 7 ^c	UD	10 $\pm$ 0.3		119 $\pm$ 36
–	Vehicle i.p.	51 $\pm$ 35 ^b	64 $\pm$ 7 ^c	UD	7 $\pm$ 1.0		87 $\pm$ 21
+	Vehicle i.n.	409 $\pm$ 58	202 $\pm$ 64	89 $\pm$ 23	7 $\pm$ 2		195 $\pm$ 79
+	Vehicle i.p.	505 $\pm$ 62	261 $\pm$ 79	80 $\pm$ 22	7 $\pm$ 0.5		136 $\pm$ 45
+	BCG i.n.	162 $\pm$ 32 ^b	142 $\pm$ 30	79 $\pm$ 21	31 $\pm$ 7 ^c		234 $\pm$ 36
+	BCG i.p.	259 $\pm$ 56 ^c	147 $\pm$ 26	127 $\pm$ 23	6 $\pm$ 1.3		327 $\pm$ 65
+	<i>M. vaccae</i> i.n.	188 $\pm$ 93	110 $\pm$ 42	143 $\pm$ 26	6 $\pm$ 0.3		265 $\pm$ 70
+	<i>M. vaccae</i> i.p.	103 $\pm$ 56 ^b	63 $\pm$ 26	110 $\pm$ 23	5 $\pm$ 0.8		350 $\pm$ 76

^a IL-3 was undetectable in either BAL or serum samples; IL-5 was undetectable in serum. UD, Undetectable.^b Value of $p < 0.01$.^c Value of $p < 0.05$.

al. (35) demonstrated a lasting proliferative response and IFN- γ release in response to purified protein derivative challenge at 1 year of age in infants immunized with BCG at birth. Interestingly, a significant suppression of IL-4 was not concurrently observed (35). The data obtained in this study support this. Murphy et al. (36) present data arguing against the plasticity of Th populations after long-term (3 wk), polarized stimulation. Such in vitro experiments almost certainly oversimplify the complex, heterogeneous stimuli presented to maturing T cells in vivo and must be very

cautiously extrapolated. Nonetheless, the issue is highly relevant and ongoing studies in our lab aim to address it. Ultimately, the quality somewhat unique to mycobacterial Ags, to evade complete eradication by the immune system and thus continue to stimulate local type 1 cytokine responses, may become realized as a rather useful, slow-release “capsule” for sustained suppression of some Th2-like responses.

In the present study, mycobacterial Ags failed to suppress the local secretion of TGF- β_1 (Table III). This cytokine has been implicated in the profibrotic changes occurring in airway remodeling (37). Investigators have further linked expression of TGF- β_1 with eosinophils (38, 39) and have found significantly increased levels in BAL fluid after allergen challenge (40) in humans. Our finding of unaffected TGF- β_1 concentrations in murine BAL samples was an unexpected result in light of the suppressed eosinophil numbers. Although a complete account of the relative role that airway macrophages may play in TGF- β_1 levels, and thus remodeling, remains to be determined, it is known that these cells can produce this cytokine (41), and activated macrophages may have been an important source in our experiment. In any event, the TGF- β_1 finding in this study does encourage a more thorough examination of the effects mycobacteria may have on airway remodeling.

The blockade of eosinophils through various mechanisms has been correlated with abrogation of LAR (42) and airway hyperactivity (19). There is also considerable evidence suggesting the eosinophil product major basic protein and its antagonistic effect on inhibitory M₂ receptors may be at least partly responsible for this acute airway narrowing (43). Eosinophils, both BAL and peribronchial, were most significantly inhibited in mice treated with BCG i.n. (Table I and Fig. 5). The most dramatic reduction in both airway hypersensitivity and hyperactivity was also observed in this group (Fig. 4). These results support the hypothesis that eosinophils, and not Ag-specific B cells, are a more important target of the action of mycobacterial Ags in this mouse model of asthma.

In summary, we have shown that low, single-dose mycobacterial treatment can suppress the LAR, AHR to methacholine, and BAL IL-5 and eosinophilia in presensitized BALB/c mice without affecting serum IgE levels. While more investigation is needed to define the durability of this effect, these results support the hypothesis that BCG may be an effective immunotherapeutic agent, operating in unique ways to inhibit asthma symptoms.

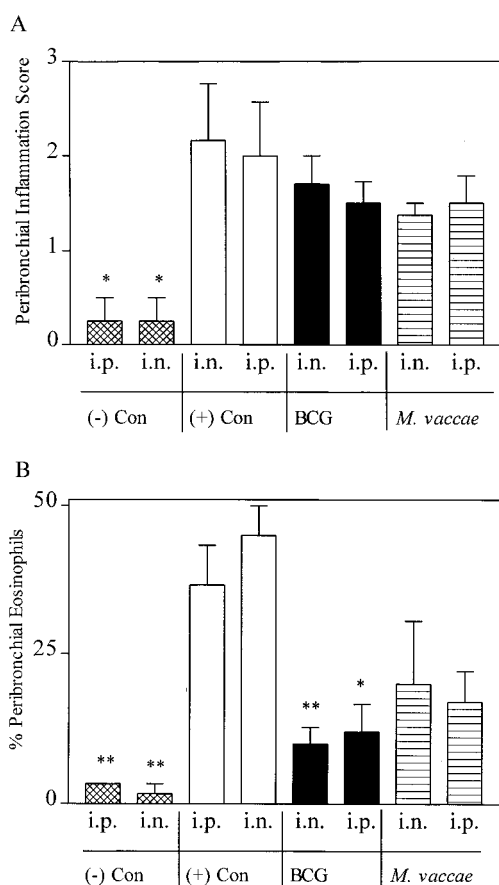


FIGURE 6. Effect of mycobacterial Ags on peribronchial cellularity and percentage of peribronchial eosinophilia in presensitized mice. *A*, Lung sections were graded as described in Fig. 2. No differences were observed between mycobacteria-treated and sensitized control groups. *B*, The percentage of eosinophils in the peribronchial inflammation was determined for each treatment group. Only BCG-treated animals showed significant protection against eosinophilic infiltration. **, $p < 0.01$; *, $p < 0.05$.

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