

ORIGINAL ARTICLE

Co-Administration of Vaccination with DNA Encoding T Cell Epitope on the Der p and BCG Inhibited Airway Remodeling in a Murine Model of Chronic Asthma

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Therapeutic modalities of airway remodeling in asthma have proved to be unsuccessful regarding reversing the previously established chronic airway changes. Recently, the potential of plasmid DNA to inhibit the Th2 immune response has been demonstrated in animal models of asthma. Bacillus Calmette-Guerin (BCG) immunization also induced immunomodulation, which appeared to be reliant on the properties of the interferon-gamma that was produced. Mice were immunized with house dust mite extract (HDM). At the 3 week point, we injected BCG subcutaneously into mice on three successive weeks. One week after the BCG injection, we immunized mice with the DNA plasmid encoding for murine T-cell epitope on Dermatophagoides pteronyssinus 2 thrice weekly. At 9 weeks after immunization, we measured airway responsiveness. Twenty four hours later, we performed bronchoalveolar lavage and histological examinations. Co-administration of DNA vaccination and BCG resulted in a partial suppression of the overproduction of goblet cells and the thickness of the peribronchial smooth muscle in ongoing allergic responses. In the bronchoalveolar lavage fluid, the number of total cells and eosinophils was reduced, and regarding the change of cytokines, the concentration of IL-4 was also decreased, but interferon-gamma was increased in the co-administration group, opposed to the asthma group. These results suggest that co-administration of vaccination with the DNA encoding T-cell epitope and BCG are effective regarding ongoing allergic response and might constitute an ideal method for combating allergic disease in the future.

Keywords asthma, DNA, house dust mite, vaccination

INTRODUCTION

Bronchial asthma is a chronic airway inflammatory disease (1, 2). Chronic exposure to allergens triggers a distinct array of immunobiological and biochemical reactions, which directly stimulate and induce abnormalities in the airway structure, resulting in the development of clinical symptoms (3, 4). The airway remodeling that occurs during the initiation of tissue repair in response to continuous allergic airway inflammation is characterized by an increase of goblet cells, hyperplasia of the mucous glands, thickening in the basement membrane due to the deposition of collagen I, III, V, fibronectin, and tenascin, alteration of the composition of the extracellular matrix (ECM), and hyperplasia and hypertrophy of the smooth muscle fibers (5, 6).

Although airway remodeling is clinically relevant, the mechanism underlying airway remodeling regarding the airway epithelial cells, inflammatory cells, and cytokines has yet to be elucidated. Therefore, the effects of pharmacological treatment on airway remodeling remain poorly understood, and this area requires a great deal more research before important questions can be satisfactorily put to rest (7). To date, it has been established that epithelial damage and Th2 cytokines act in concert, inducing a functional disturbance

of the epithelial-mesenchymal trophic unit, resulting in myofibroblast activation and the inflammatory and remodeling responses characteristic of chronic asthma (6). Consequently, new immunotherapeutics, exhibiting less severe side effects and higher efficacy and targeted toward the reprogramming of immune response towards a Th1 pathway, represent the most promising extant approaches for the treatment of airway remodeling.

Within the context of allergy, the administration of DNA encoding for allergens can selectively prevent Th2 cell activation and can shift typical Th2 allergic responses to Th1 when used on allergic animals (8). Moreover, the injection of plasmid DNA encoding for T-cell epitopes can suppress allergic reactions as effectively as the injection of whole segments of the encoding region of the allergen (9). However, the use of an appropriate adjuvant is pivotal in optimizing the response to DNA vaccines, due primarily to the relatively low efficacy of DNA vaccines regarding the induction of immune responses (10). BCG contains both adjuvant and antigen components capable of stimulating CD8+ T cell generation that secrete the interferon-gamma (IFN- γ) maintaining the dominance of Th1-type immune response (11).

We have developed a murine model of allergic asthma, in which BALB/c mice are sensitized to house dust mites (HDM) and then repeatedly exposed to HDM intranasally. In this model, several symptoms can be observed that resemble those found in patients with allergic asthma, including the presence of immunoglobulin E (IgE) in the serum, increased airway responsiveness, airway inflammation, and remodeling.

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The aim of this study was to investigate the effects of co-administering vaccination with DNA encoding for T-cell epitope on Dermatophagoide pteronyssinus (Der p) and Bacillus Calmette-Guerin (BCG) inhibited airway remodeling in ongoing allergic responses. We assessed the pattern, as well as the degree, of the airway remodeling and the alteration of inflammatory cells and cytokines in the bronchoalveolar lavage after treatment.

MATERIALS AND METHODS

Experimental Animals and Experiment Group

A total of 60 Balb/C female mice, 4 to 6 weeks of age were used. Animals were raised in the animal facility at the clinical research center of the Catholic University Medical School. They were maintained and used according to the protocols provided by the internal review board of the clinical research center at the Catholic University Medical School.

Immunization and Administration of HDM by Inhalation to Mice

Der p (Bencard, Brentford, UK) was emulsified with an equal volume of complete Freund's adjuvant (CFA, Imject Freund's complete adjuvant; Pierce, IL, USA) for immunization. Mice were sensitized subcutaneously at the base of the tail with 100 μ g of Der p extract in CFA. The mice were also given an intraperitoneal dose of 300 ng of purified pertussis toxin (Sigma-Aldrich, St. Louis, MO, USA) at 24 and 72 hours after initial immunization. Seven days later, the mice were again boosted with the same amount of antigen in incomplete Freund's adjuvant (Imject Freund's incomplete adjuvant; Pierce, IL). Mice were given 10 μ g of Der p extract via intranasal inhalation six times at weekly intervals from the booster until 6 weeks, and followed by 2 days/week, for up to 3 consecutive weeks. At 9 weeks after immunization, these animals were further exposed to 10 μ g of Der p extract intranasally for 3 consecutive days. A control group ($n = 15$) was sensitized and challenged on an identical schedule except for the intranasal inhalation with PBS (Figure 1).

Plasmid Construction and DNA Preparation

RNA was isolated from HDM, according to the instructions for the Perkin-Elmer Gene Amp RNA PCR kit (Perkin

Elmer, Branchburg, NJ, USA) and used as a template for reverse transcriptase. This cDNA was then used to carry out PCR with Taq polymerase, using primers specific for the Der p2 epitope region, 87–129 (5'-CCGGAATTCGCC GCCACCATGGATATTAAATATACATGGAATGTTCCGA -3' and 5'-TGCTCTAGATTAATCGCGGATTTTAGCATGA GTAGC-3'). This primer encompasses the murine T-cell epitope in Der p 2 (12) and also includes EcoR1 and Xba1 sites for cloning. The amplified PCR products were then cloned into pcDNA3.1 eukaryotic expression vector (Invitrogen, San Diego, CA, USA) and sequenced to verify the insertion of the right gene with the appropriate open reading frame. Each plasmid construct was prepared with Maxi Prep (Quiagen, Chatsworth, CA, USA).

DNA Vaccination and BCG Immunization

The vaccination group ($n = 15$) was vaccinated by injection with 100 μ g of naked DNA encoding for the murine T-cell epitope on Der p 2, in 100 μ l PBS three times at weekly intervals into muscles, after 4 weeks of immunization with Der p. The co-administration group was subcutaneously immunized with 2×10^4 colony-forming units (CFU)/100 μ l of BCG (Pasteur 1173P; Korean National Tuberculosis Association, Seoul, Korea) three times weekly, beginning 3 weeks after sensitization with HDM before DNA vaccination. The asthma group ($n = 15$) was treated with PBS, rather than DNA vaccination.

The Assessment of the Total Serum IgE Antibody Response

Blood samples from 15 mice in each group were collected four times, on weeks 0, 3, 6, and 9. The total level of IgE was detected by enzyme linked immunosorbent assay (ELISA) as follows. A total of 100 μ L of anti-mouse immunoglobulin E (IgE) capture mAb (clone R35-72; Pharmingen, San Diego, CA, USA) was added in each well to plate and was incubated overnight at 4°C. After blocking with PBS + 10% fetal calf serum, the mouse sera were incubated overnight at 4°C, and antibody binding was assessed by the addition of 100 μ L of HRP-conjugated anti-mouse IgE detection mAb (clone R35-118; Pharmingen, San Diego, CA) overnight at 4°C. After adding the enzyme substrate, the plates were read at 492 nm in an ELISA reader (Bio-Rad, Richmond, CA, USA). The

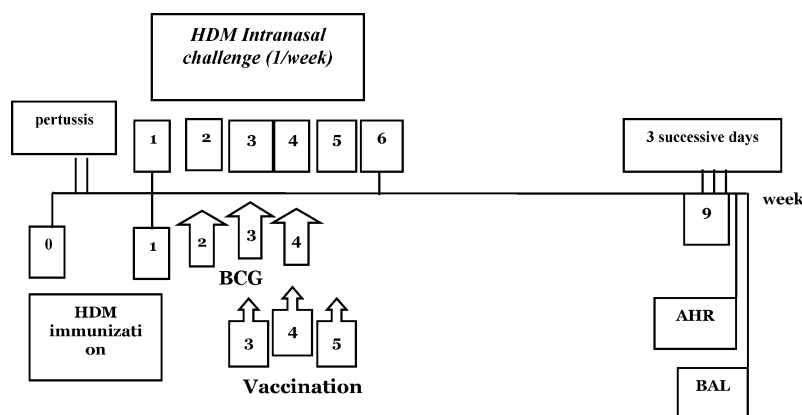


FIGURE 1.—Immunization scheme for treatment of airway remodeling by co-administration of DNA vaccination and BCG.

purified mouse anti-IgE antibody (Pharmigen, San Diego, CA, USA) was used as a total IgE standard.

Determination of HDM-Specific IgG1 and G2a Antibody Response (13)

HDM-specific IgG1 and G2a levels were determined by ELISA as follows: 50 μ L of Der p (5 μ g/mL in 0.1 M carbonate buffer, pH 9.6) was dispensed into each well of a polystyrene microtiter plate (Cost, Cambridge, MA, USA), and incubated overnight at 4°C. The antigen-coated plates were washed three times with 0.05% PBS-Tween 20 buffer (washing buffer) and incubated with mouse sera overnight at 4°C. Plates were washed five times with washing buffer and incubated with peroxidase-conjugated anti-mouse IgG1 or G2a antibody (Sigma, St. Louis, MO, USA) overnight at 4°C and then washed five more times before the addition of citric acid-phosphate buffer (pH 5.0) containing 0.15 mg/mL of O-phenylenediamine (Sigma, St. Louis, MO). Color was developed at room temperature, and the reaction was halted with 2.5M sulfuric acid and measured at 492 nm (Bio-Rad, Richmond, CA). The antibody production units (APU) of the antigen-specific antibodies were determined by comparing a plot of absorbance versus dilutions of the sample to that of the standard versus its concentration.

Measurement of Airway Responsiveness

Twenty four hours after the last challenge, mice were placed in a barometric plethysmographic chamber (OCP 2000; Allmedicus, An Yang, Korea). Methacholine was used at concentrations of 3.125 mg/mL, 6.25 mg/mL, 12.5 mg/mL, and 50 mg/mL. A total of 3 mL of each concentration was inhaled for 3 minutes as an aerosol, prepared by an Ultra-Neb (3650p, DeVilbiss, Pennsylvania, PA), after which time the Penh (enhanced pause) value was measured for 3 minutes. Bronchopulmonary resistance was expressed as enhanced pause (Penh), which was calculated as follows, according to the manufacturer's protocols:

$$\text{Penh} = (\text{Expiratory time/Relaxation time} - 1) \times (\text{Peak expiration flow/Peak inspiration flow})$$

Results are expressed as percentage increases in Penh after challenge with each concentration of methacholine, where the baseline Penh (after saline challenge) equals as 10%.

The Analysis of the Bronchoalveolar Lavage Fluid

Twenty four hours after the assessment of airway responsiveness, the left major bronchus was tied with a string, a 22-gauge needle was inserted, and the bronchoalveolar lavage fluid was obtained by the infusion and collection of 0.4 mL PBS, repeated 3 times. The bronchoalveolar lavage fluid was centrifuged at 4°C, 100 g, for 7 minutes, and the supernatant was stored at -70°. The pellet was resuspended in 1 mL PBS containing 10% FBS (GibcoBRL, Life Technologies, Gaithersburg, MD, USA), and the total cell number was counted by mixing the 100 μ L supernatant with 300 μ L Trypan blue (Gibco, Grand Island) using a hemacytometer. The differential calculations of the number of alveolar macrophage, eosinophils, lymphocytes, and neutrophils were conducted by adjusting the cell number to 1×10^6 cells/mL,

preparing slides with a cytocentrifuge (Cytospin 2, Shandon, Runcorn, UK), staining with Diff-Quik, and counting at least 500 cells under 400 X magnification.

The Assessment of Cytokines in the Supernatant of the Bronchoalveolar Lavage fluid

The concentrations of IL-13, IL-4, IL-10, IFN- γ , and transforming growth factor- β (TGF- β) (R&D system, Minneapolis, MN, USA) in the bronchoalveolar lavage fluid were measured using ELISA kits. The minimum detectable concentrations were 5, 2, 4, 2, and 7 pg/mL, respectively.

The Assessment of Airway Remodeling

The left side lung, which had not undergone bronchoalveolar lavage, was resected, fixed immediately in 4% paraformaldehyde, and used to prepare tissue-sections. To determine the increase of goblet cells in the bronchial epithelium, periodic acid-Schiff (PAS) staining was performed. The degree of goblet cell increase was assessed according to the 5-point scoring system, which classifies according to the proportion of goblet cells in the epithelium of the entire bronchus. Grade 0, the absence of goblet cells; grade 1, less than 25%; grade 2, 25~50%; grade 3, 50~75%, and grade 4, 75~100%, or the obstruction of the bronchus due to mucus (14). To determine the degree of subepithelial fibrosis and peribronchial smooth muscle layer, Masson's Trichrome staining was performed. We measured both the stained area of the subbasement membrane due to fibrosis and the stained area due to smooth muscle (15). The average value for the entire bronchus was obtained, based on the circumference of the basement membrane, by selecting 2 or 3 sections that were equivalent in size and shape. All measurements were performed with the computerized image analyzer program (BX50; Olympus, Tokyo, Japan).

Statistical Analysis

The data were presented as the mean $\pm$ standard deviation. The statistical analysis of parameters was performed by an independent sample *t* test using the software for IBM-PC (SPSS for Windows version 10.0). *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

The Total Serum IgE and HDM-Specific IgG1 and G2a Response After Co-Administration of DNA Vaccination and BCG

There was no change in total serum IgE responses between groups until 6 weeks after induction by HDM through the nose. Nine weeks after priming, the total serum IgE responses in the co-administration group ($3.46 \pm 0.50 \mu\text{g/mL}$) and the vaccination group ($3.68 \pm 0.58 \mu\text{g/mL}$) exhibited significantly more suppression than did the asthma group ($5.411 \pm 1.27 \mu\text{g/mL}$) ($p < 0.01$). However, BCG did not exhibit any additive effect from DNA vaccination on the total IgE response (Figure 2A). Nine weeks after the immunization, HDM-specific IgG2a response, which indirectly reflects the effect of IFN- γ , was higher in the co-administration group ($8.71 \pm 1.22 \times 10^6$ APU) and vaccination group ($8.89 \pm 1.13 \times 10^6$ APU) than in the asthma group ($7.45 \pm 0.82 \times 10^6$ APU) ($p < 0.01$, Figure 2B). Conversely, HDM-specific

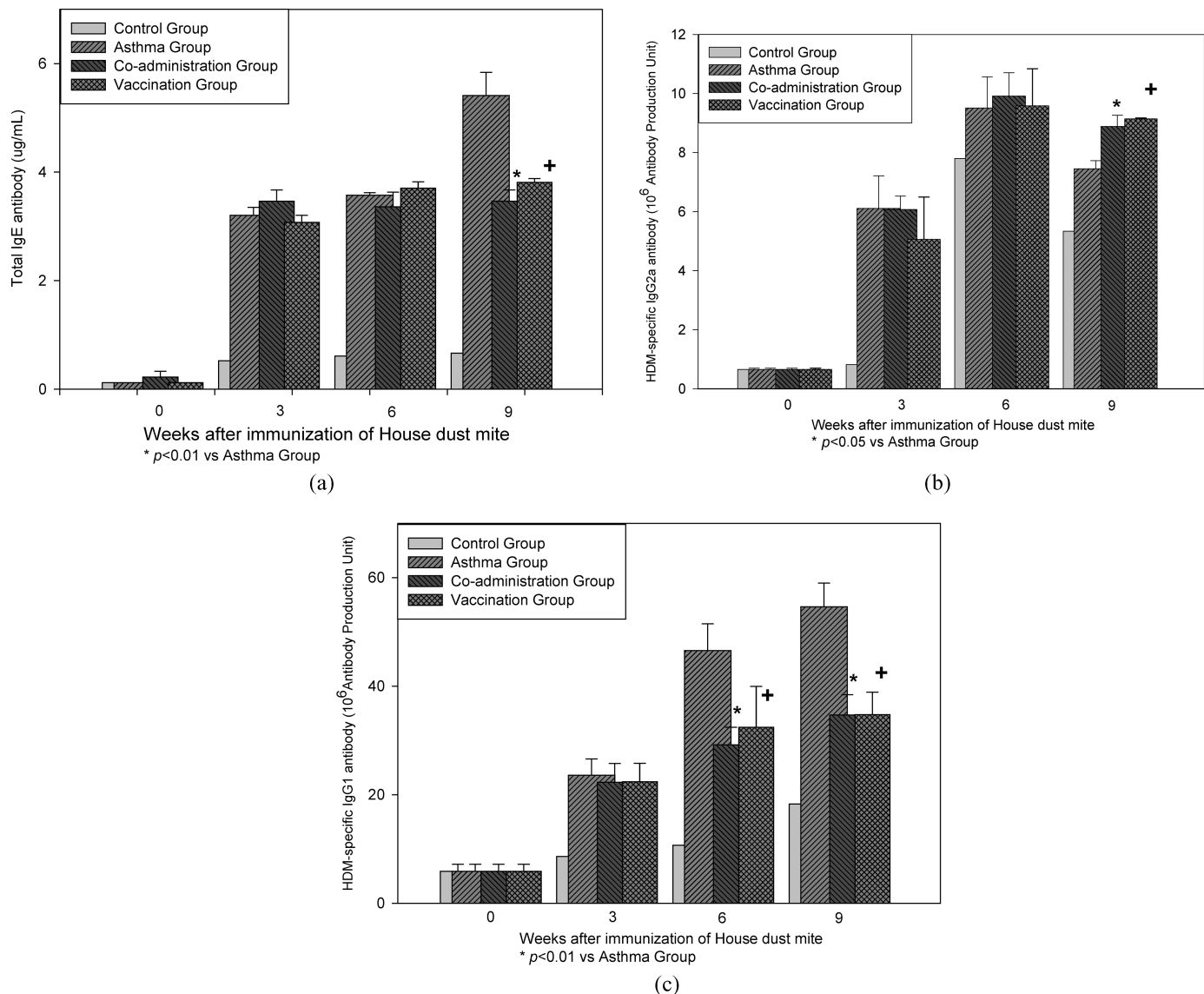


FIGURE 2.—The effect of co-administration of DNA vaccination and BCG on the total serum IgE (A), HDM-specific IgG2a (B), and HDM-specific IgG1 (C) antibody responses. Data are represented as means $\pm$ SD ($n = 15$), * $p < 0.01$ co-administration group compared with asthma group. + $p < 0.01$ vaccination group compared with asthma.

IgG1 response, which indirectly reflects the effects of IL-4, was attenuated more severely in the co-administration ($26.16 \pm 4.58 \times 10^6$ APU) and vaccination groups ($34.39 \pm 9.62 \times 10^6$ APU) than in the asthma group ($46.53 \pm 11.08 \times 10^6$ APU) at 6 weeks, and these levels persisted until 9 weeks (Figure 2C). However, no difference was seen in the effects on HDM-specific IgG2a and IgG1 responses between the co-administration and vaccination groups.

The Change of Inflammatory Cells in the Bronchoalveolar Lavage Fluid

The total cell number in the bronchoalveolar lavage fluid of the co-administration ($7.57 \pm 3.20 \times 10^5$ /mL) and vaccination group ($8.34 \pm 4.50 \times 10^5$ /mL) at 9 weeks after immunization was significantly lower than in the asthma group ($14.81 \pm 3.50 \times 10^5$ /mL) ($p < 0.05$, Figure 3A). The number of eosinophils in the co-administration ($2.63 \pm 2.25 \times$

10^4 /mL) and vaccination groups ($5.40 \pm 4.50 \times 10^4$ /mL) was reduced more than in the asthma group ($47.50 \pm 15.73 \times 10^4$ /mL) ($p < 0.05$, Figure 3B).

The Evaluation of Cytokines in the Supernatant of the Bronchoalveolar Lavage Fluid

To determine the change in cytokines, cytokines in the supernatant of the bronchoalveolar lavage fluid were measured 9 weeks after immunization. Co-administration and vaccination groups exhibited higher IFN- γ levels than the asthma group (14.11 ± 3.11 pg/mL, 13.42 ± 2.68 pg/mL vs 8.92 ± 0.55). IL-4 levels were lower in the co-administration (12.19 ± 0.31 pg/mL) and vaccination groups (12.26 ± 1.31 pg/mL) than in the asthma group (17.84 ± 1.16) ($p < 0.05$). By way of contrast, IL-10, IL-13, and TGF- β levels were comparable to those observed in the asthma group (Table 1).

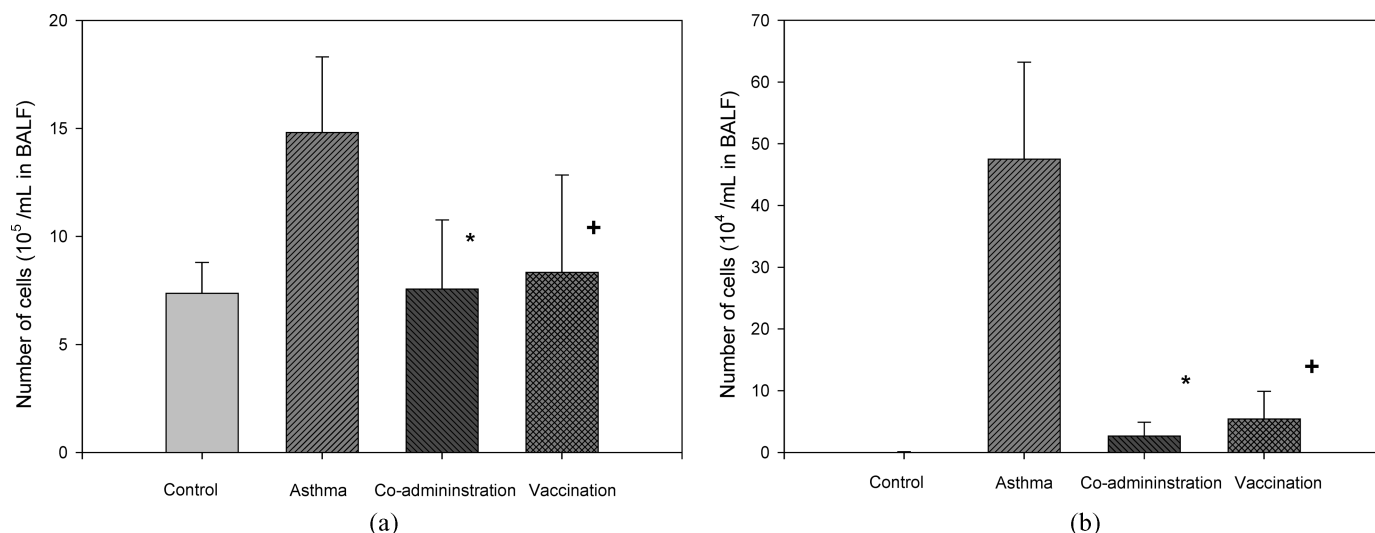


FIGURE 3.—The number of inflammatory cells (A) and eosinophils (B) in bronchoalveolar lavage fluid at 9 weeks after systemic immunization. Data are represented as means $\pm$ SD ($n = 15$), * $p < 0.01$ co-administration group compared with asthma group. + $p < 0.01$ vaccination group compared with asthma group.

Changes in Airway Responsiveness

To assess airway responsiveness, we performed a bronchial challenge test using methacholine. Figure 4 demonstrates that the sensitization of mice with HDM resulted in the development of significant airway hyperresponsiveness (AHR) to methacholine challenge. The co-administration and vaccination group exhibited a dramatic attenuation in the degree of AHR.

The Degree of the Change of the Airway Remodeling

On the Periodic Acid-Schiff (PAS) staining of lung tissues, vaccination and co-administration groups were found to have lower numbers of goblet cells than the asthma group. According to the results of Masson's Trichrome staining, the peribronchial smooth muscle were thickened to a lesser degree in the vaccination and co-administration groups at 9 weeks after systemic immunization with HDM than were those of the asthma group (Figure 5). Measuring the increase of goblet cells according to the aforementioned 5-point scoring system, the degree of goblet cell proliferation was lower in the vaccination (1.48 ± 0.77) and co-administration groups (1.47 ± 0.69) than in the asthma group (3.80 ± 0.62), but was not completely abrogated in any group. By way of contrast, this increase was not detected in the control group (Figure 6A). According to the results of Masson's Trichrome staining, the peribronchial smooth muscle layers of the vaccination ($19.96 \pm 7.36 \times 10^4 \mu\text{m}^2$) and co-administration

groups ($17.43 \pm 6.21 \times 10^4 \mu\text{m}^2$) were thickened to a significantly lower degree than those of the asthma group ($29.11 \pm 8.14 \times 10^4 \mu\text{m}^2$) ($p < 0.05$, Figure 6B). However, the vaccination and co-administration groups exhibited no differences from the asthma group regarding the degree to which subepithelial fibrosis occurred (Figure 6C). Furthermore, no differences in airway remodeling were evident between the vaccination and co-administration group.

DISCUSSION

Allergic bronchial asthma is a chronic disease caused by repeated exposures to an allergen in which the chronic inflammatory condition in the airway induces structural abnormality, eventually leading to the development of airway remodeling. Hyperplasia of the goblet cells and structural changes

TABLE 1.—Effect of Co-administration of BCG and DNA vaccination on cytokine levels in bronchoalveolar lavage fluid.

	Control	Asthma	Coadministration	Vaccination
IL-4 (pg/mL)	14.15 $\pm$ 1.64	17.84 $\pm$ 1.16	12.19 $\pm$ 0.31*	12.26 $\pm$ 1.31*
IL-13 (pg/mL)	18.30 $\pm$ 2.29	21.24 $\pm$ 5.91	18.35 $\pm$ 4.03	17.19 $\pm$ 2.34
IL-10 (pg/mL)	18.91 $\pm$ 0.90	24.04 $\pm$ 3.14	21.29 $\pm$ 0.14	20.34 $\pm$ 3.54
IFN- γ (pg/mL)	8.07 $\pm$ 0.27	8.92 $\pm$ 0.55	14.11 $\pm$ 3.11*	13.42 $\pm$ 2.68*
TGF- β (pg/mL)	3.78 $\pm$ 0.36	7.16 $\pm$ 1.96	6.54 $\pm$ 0.72	7.34 $\pm$ 3.56

Data represent means $\pm$ SD ($n = 15$).

* $p < 0.05$ compared with asthma group.

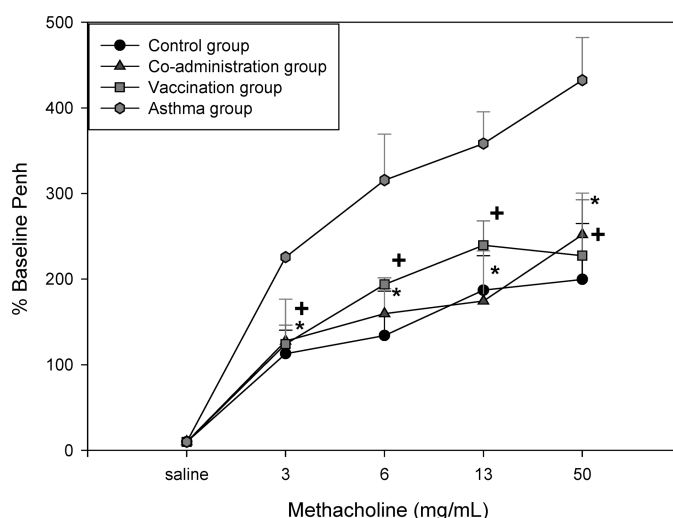


FIGURE 4.—Effect of co-administration of DNA vaccination and BCG on airway responsiveness in mice sensitized and challenged with HDM. * $p < 0.01$ co-administration group compared with asthma group; + $p < 0.01$ vaccination group compared with asthma group.

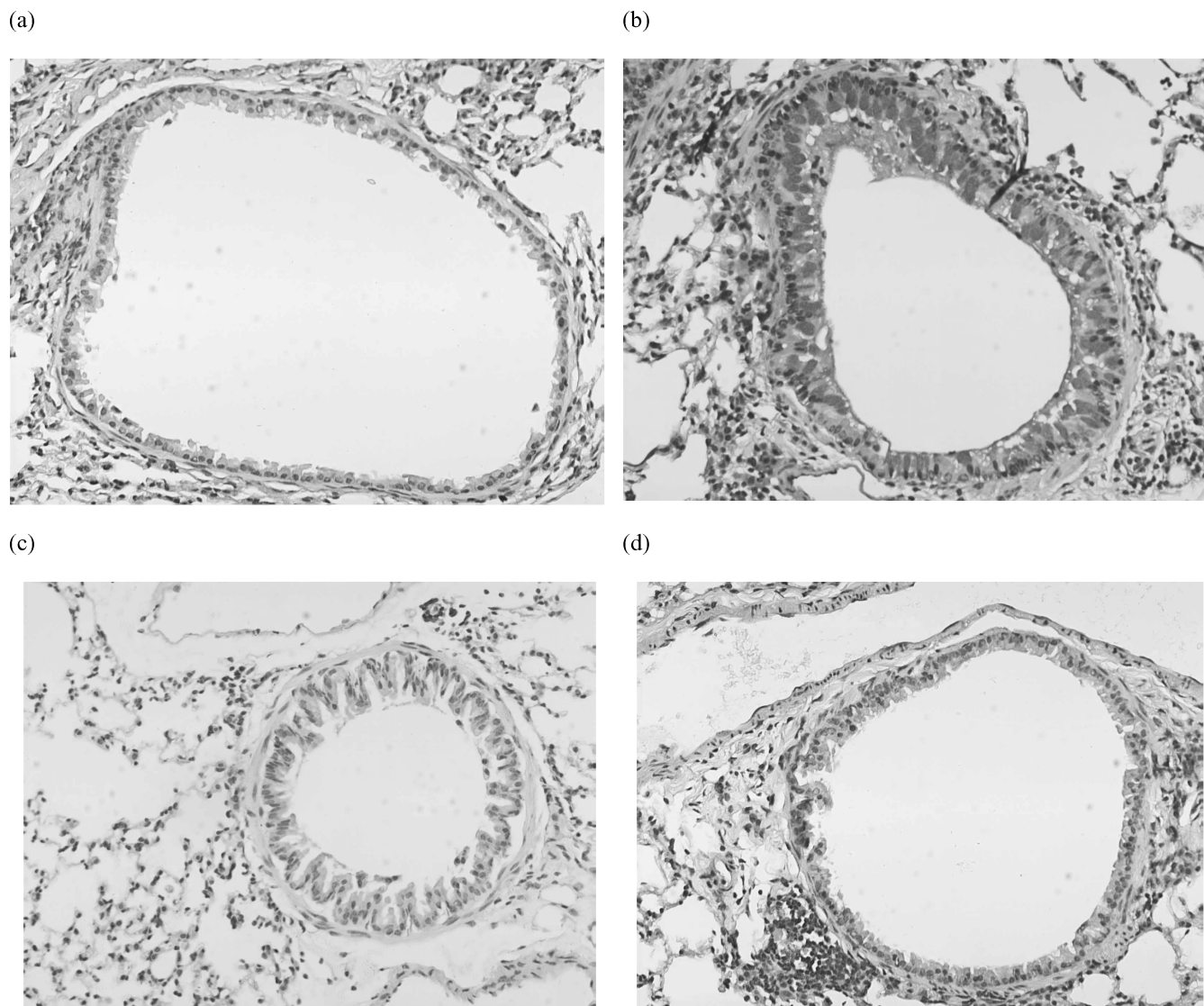


FIGURE 5.—Histological analysis of lung section. The lung sections stained with Periodic acid-Schiff (A, B, C, and D) and Masson's Trichrome (E, F, G, and H). Slides were photographed at $\times 200$ magnification. (A and E) control group, (B and F) asthma group, (C and G) co-administration group, and (D and H) vaccination group.

in the subbasement membrane induce functional impairment, resulting in the appearance of typical clinical symptoms, including bronchial hypersensitivity and airway constriction (16, 17). Clinically, although the prevention or treatment of airway remodeling in early stages may facilitate the effective resolution of asthma, therapeutics that improve airway remodeling are, at present, quite scarce (18, 19). The results of this study demonstrate that co-administration of vaccination with the DNA encoding for T-cell epitope and BCG ameliorates the factors relevant to airway remodeling, the degree of goblet cell numbers, and peribronchial smooth muscle thickening in murine asthma ongoing allergic responses. This treatment also resulted in the inhibition of allergic inflammatory responses and airway responsiveness.

Airway remodeling is a repair phenomenon against chronic airway inflammation and damage. Due to various mediators involved in this process, the mechanisms that maintain nor-

mal airway conditions are disrupted, resulting in the appearance of hypertrophy of the smooth muscles, fibrosis of the subbasement membrane, hyperplasia of goblet cells, etc. The increased smooth muscle volume, caused by the hyperplasia of the smooth muscles, results in a thickening of the airway wall surrounding the airway lumen, thereby decreasing the diameter of the airway lumen. This mechanism plays an important role in the development of hyperresponsiveness (5). In addition, the hyperplasia of the goblet cells occurring in chronic asthma increases mucous secretion in the epithelium of the airway, increases the sputum volume clinically, decreases the diameter of the airway lumen, and thus exacerbates asthma (17). Furthermore, the cytokines involved in the immune response of type 2 helper cells, including IL-13, IL-4 and TGF- β , mediate their effects on the epithelial cells in the airway, induce the hyperplasia of goblet cells, simultaneously induce fibrosis in the subbasement membrane, and thus are

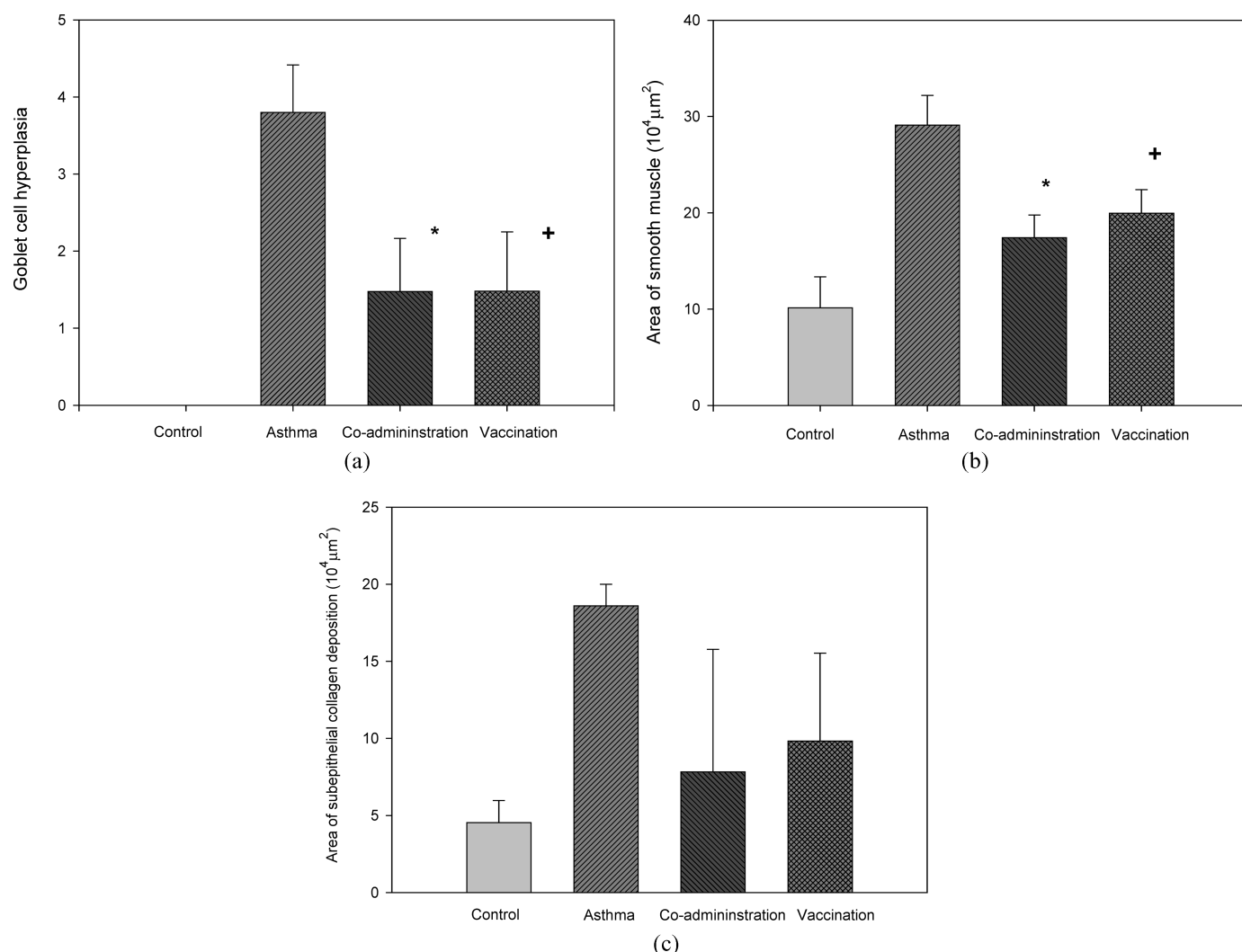


FIGURE 6.—Morphometric analysis of lung histology. The ordinates indicate the score of hyperplasia of the goblet cells (A). The extent of peribronchial smooth muscle (B) and subepithelial fibrosis (C) in each group. Data are represented as the means $\pm$ SD ($n = 15$), * $p < 0.05$ co-administration group compared with asthma group; + $p < 0.05$ vaccination group compared with asthma group.

deeply involved in the alteration of ECM components (20). As Th2-type cytokines play a major role in the pathogenesis of airway remodeling, the treatment of remodeling may induce a Th2 to Th1 cytokine shift or augment Th1 cytokines. The co-administration and vaccination group resulted in a suppression of the numbers of eosinophils and of IL-4 production in BAL fluids, and augmented the concentration of IFN- γ in the BAL fluid (Table 1). This is consistent with reports that DNA vaccination induced IFN- γ production in a murine asthma model (7, 8) and that the production of IFN- γ during an active BCG infection blocks the expansion of Th2 secreting IL-4 in the lung (21, 22). The co-administration of DNA vaccination and BCG has been suggested to suppress IL-4 production and the number of eosinophils in BAL fluid, owing to the induction of the IFN- γ inhibited IL-4-induced effect on T cells, thereby inducing a shift to a Th1-type immune response. Although the co-administration of DNA vaccination and BCG did not directly affect the production of IL-13 or TGF- β , it might skew the Th1/Th2 balance toward a Th1-type immune response, thereby reducing the degree of goblet cell hyperplasia

and the extent of peribronchial smooth muscle thickening. However, after treatment, we observed no changes in the extent to which subepithelial fibrosis occurred. It was previously reported that mice that were treated with CpG DNA at the time of sensitization had significantly fewer features of airway remodeling, including reduced thickness of the subepithelial collagen layer, lower total lung collagen content, and less goblet cell hyperplasia. However, this study has shown that CpG oligodeoxynucleotides (ODNs) may only prevent chronic inflammatory structural changes in the airways seen in chronic asthma (23). Because sensitization has occurred in most asthmatic subjects before clinical presentation with the disease, we examined the ability of DNA vaccination to reverse allergic responses once they have been established in the lung. In other reports, BCG immunization exerts a preventive effect, not only on goblet cell hyperplasia and subepithelial smooth muscle thickness, but also in the subepithelial fibrosis of bronchial airways (11). The differences observed in our results might be attributable to differences in BCG dosage. In these other studies, the researchers used

500 times more BCG than was designated in our protocols. However, other reports have also demonstrated that the use of 2×10^4 CFU of BCG exerts the same suppressive effects on allergic inflammation (22). These doses of BCG are sufficient dose for the preventive effects on allergic inflammation but could not be capable of the suppressive effects on the subepithelial fibrosis of asthmatic mice in ongoing allergic inflammation. 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 late asthmatic response (LAR), and eosinophilia (24). Recently, via the release of TGF- β and its effects on underlying fibroblasts and myofibroblasts, remodeling signals initiated in the repairing epithelium have been propagated (6). The co-administration and vaccination group in our study could not inhibit the production of TGF- β during ongoing allergic responses. In view of these reports, the most likely explanation for our results is that TGF- β plays a major role in the pathogenesis of subepithelial fibrosis.

After the co-administration of DNA vaccination and BCG, total serum levels of IgG2a-anti-HDM antibody were elevated, and HDM-specific IgG1 responses were reduced. IgG2a is dependent on IFN- γ as an IgM-to-IgG2a switching factor and is believed to be a component of Th1 response (25). By way of contrast, IgG1 relies on the IL-4 that is secreted by the Th2 cells. This suggests that co-administration of DNA encoding for human T-cell epitope and BCG would induce high levels of IFN- γ and IgG2a antibody production, thereby indicating that genetic immunization suppresses IgE production via the induction of Th1 response from the T helper cells. The most likely explanation for our results is that plasmid DNA, including CpG-ODN, induce Th1 response via the secretion of IFN- γ and IL-12, or directly suppress the production of IgE in B cells, which opposes the established Th2 allergic response.

In our study, BCG was shown to exert a few additional effects regarding genetic vaccination in ongoing allergic inflammatory responses. It has been reported that the co-injection of Amb a 1 with ISS-ODN also induced a TH1 response in mice (26). However, this was not an additive effect. In addition, a preventive effect has been observed on various parameters of allergic inflammation in mouse models after pretreatment, but it is difficult to attenuate a preexisting allergic state. In light of the potential applications of the literature in this area, our finding regarding the effects of co-administration of DNA vaccination and BCG on allergic response in a preexisting allergic state becomes important.

In conclusion, our data demonstrate that the co-administration of DNA vaccination with BCG suppressed the degree to which goblet cells were overproduced, as well as the thickness of peribronchial smooth muscle during ongoing allergic responses. In the bronchoalveolar lavage fluid, the number of total cells and eosinophils was reduced. Regarding changes in cytokines, the concentration of IL-4 was reduced, but the IFN- γ concentrations increased, in the co-administration group, as opposed to the asthma group. Envisioning future immunotherapies for allergic asthma must certainly take into account presensitized individuals, in addition to attempting to prevent sensitization altogether. Co-administration of the vaccination with the DNA encoding for T-cell epitope and BCG are effective regarding ongoing

allergic responses and might constitute an ideal method for combating allergic disease in the future.

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