

Prolonged Antigen Exposure Ameliorates Airway Inflammation But Not Remodeling in a Mouse Model of Bronchial Asthma

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Key Words

Cytokines · IL-12 · Th1 · Th2 · Tolerance

Abstract

Background: In naive rodents, repeated exposure to aerosolized antigen induces suppression of the Th2 response to the antigen. We hypothesized that more prolonged exposure of established asthma model to antigen aerosols may downregulate asthmatic phenotype. **Methods:** After establishing an ovalbumin (OVA)-induced asthma model, mice were further exposed to OVA (prolonged exposure group) or phosphate-buffered saline (positive controls) 3 days per week for 6 weeks. During week 7, the mice of both groups were finally challenged with OVA. **Results:** Prolonged OVA exposure resulted in marked suppression of serum OVA-specific immunoglobulin E (IgE) antibody levels, eosinophilia of the airway, and airway hyperresponsiveness (AHR). However, airway remodeling characterized by goblet cell hyperplasia and airway fibrosis was observed to the same degree in both groups. These effects were accompanied by diminished production of Th2 cytokines such as interleukin-4 (IL-4), IL-5 and IL-13 in bronchoalveolar lavage fluid (BALF) and cultured supernatant of splenocytes. Furthermore, prolonged exposure markedly increased IL-12 lev-

els in BALF. **Conclusions:** Prolonged antigen exposure has inhibitory effects on eosinophilic inflammation, AHR and IgE response to antigen, but not on airway remodeling, presumably via inhibition of Th2 cytokines and increased IL-12 production in the lungs.

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Introduction

Common environmental allergens naturally sensitize atopic individuals to produce IgE class antibody via airway mucosa. Susceptibility to such sensitization is called 'atopy' and is genetically determined [1]. Atopy is frequently found in association with bronchial asthma. However, its pathophysiology remains largely unknown. Inhalation of allergens in patients with atopic asthma induces airway hyperresponsiveness (AHR) and inflammation. Persistence of airway inflammation in patients with atopic asthma seems to be due to continuous exposure to the allergens, since avoidance of allergens improves inflammation [2]. It is also known that patients with atopic or occupational asthma who continue to be exposed to specific allergens may experience progressive airflow limitations possibly due to airway remodeling [3–5]. On the other hand, nonatopic individuals do not

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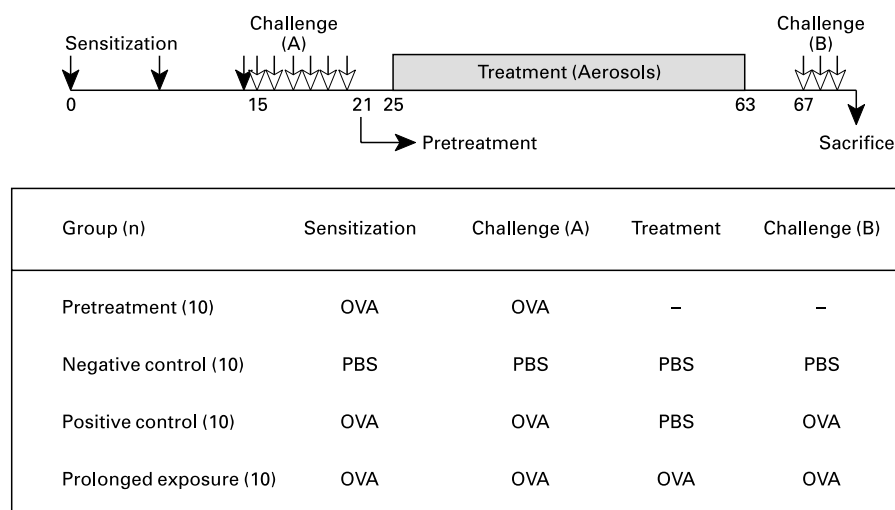
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Fig. 1. Experimental protocol of antigen exposure. Mice were sensitized with OVA plus alum 3 times at weekly intervals, and then challenged with aerosolized OVA (5%) for 6 consecutive days. Pretreatment group mice were examined on day 21. Following pretreatment procedures, mice were divided into two treatment groups. Prolonged exposure group mice were repeatedly exposed to 5% OVA aerosol on 3 consecutive days per week for the next 6 weeks. Positive control group mice were exposed to PBS alone. Mice of both groups were finally challenged with 5% OVA aerosol for 3 days. Negative control group mice were sensitized, challenged and treated with PBS alone.



develop airway inflammation in spite of inhalation of the same environmental allergens. The mechanisms that prevent allergen-induced airway inflammation in nonatopic individuals are not known.

In rodents, repeated exposure to aerosolized antigen induces suppression of the Th2 response to the antigen [6]. This suppression is transferable by T cells, of which the phenotype is controversial at present. The regulatory cells were reported to be interferon- γ (IFN- γ)-producing CD8+ and TCR- $\gamma\delta$ T cells [7–9], whereas a study with knockout mice showed that the suppression does not require CD8+ cells, TCR- $\gamma\delta$ T cells or IFN- γ [10]. In any case, the existence of aerosolized antigen-induced tolerance indicates the existence of mechanisms for downregulation of the Th2-type host response to inhaled antigens.

We hypothesized that a more prolonged exposure of the established asthma model to antigen aerosols may downregulate the asthmatic phenotype. In the present study, we examined the effects of prolonged exposure to aerosolized ovalbumin (OVA) on the OVA-induced mouse asthma phenotype. Prolonged antigen exposure of the model mice to aerosolized OVA induced marked suppression of eosinophilic inflammation, AHR to methacholine (MCh), and immunoglobulin E (IgE) antibody response. However, airway remodeling was not suppressed by prolonged exposure to antigen aerosols. To investigate the mechanism of these effects, we also studied the concentration of cytokines in bronchoalveolar lavage fluid (BALF) and the supernatant of OVA-stimulated splenocytes.

Materials and Methods

Animals and Experimental Protocol

The specific pathogen-free BALB/c mice (6 to 8-week-old females) were obtained from Charles River Laboratories (Kanagawa, Japan). Mice were primed intraperitoneally with 10 μ g OVA (grade V; Sigma Chemical Co., St. Louis, Mo.) and 20 mg Al(OH)₃ in 0.2 ml of phosphate-buffered saline (PBS, pH 7.4) once per week for 3 weeks. Twenty-four hours after the last injection, the primed mice were placed individually in a 50-ml plastic tube and challenged by exposure to aerosolized 5% OVA/PBS, which was delivered by a DeVilbiss 646 nebulizer (DeVilbiss, Somerset, Pa.) driven by compressed air at 5 liters/min for 20 min. Challenge was carried out once a day for 6 consecutive days (days 15–20). On day 21, 10 mice were analyzed for AHR, cell number and differentials in BALF, and circulating OVA-specific antibody levels (fig. 1; pretreatment stage). Marked eosinophilic inflammation and AHR are generally induced by this treatment [11, 12].

Four days after the final challenge, the other set of mice was divided into two groups (treatment groups). As a method for prolonged exposure, we used aerosolized OVA of a fixed concentration (5%); i.e., one group (n = 10, prolonged exposure group) was exposed repeatedly to 5% OVA aerosols (20 min) on 3 consecutive days per week for 6 weeks (fig. 1). The other group (n = 10, positive control group) was exposed only to aerosolized PBS in the same manner for 6 weeks. Beginning on day 67, both groups were challenged with 5% OVA aerosols for 3 consecutive days. For a negative control, 10 mice were sensitized, challenged and treated with PBS alone. All mice were killed on day 70.

Measurement of AHR, Analysis of BALF and Determination of Serum-Specific Antibody Levels

AHR was measured 24 h after the final aerosol challenge (day 21 for the pretreatment group and day 70 for the treatment and negative control groups) while conscious and under unrestrained conditions by whole-body plethysmography (Buxco, Troy, N.Y.) as described

previously [11]. Mice were killed by cervical dislocation under anesthesia after measurement of AHR. BALF was obtained by injections of 0.5 ml PBS 3 times (total 1.5 ml), as described previously [11]. Serum OVA-specific IgE and IgG2a antibody titers were determined by indirect ELISA [11]. As an internal standard, pooled serum from OVA-immunized BALB/c mice was included in each assay. The standard serum was assigned values of 100 and 10,000 U/ml for OVA-specific IgE and IgG2a, respectively.

Histology

Following bronchoalveolar lavage, the lungs were perfused in 10 ml PBS by puncture of the right ventricle. Lungs were removed and fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 5- μ m sections. The sections were stained with hematoxylin and eosin, elastica-Masson's trichrome, or Alcian blue at pH 2.5/periodic acid-Schiff (AB/PAS).

The number of eosinophils, goblet cells (AB/PAS-positive cells) and total epithelial cells (total number of nuclei in the epithelium) were counted using an image analysis system. The slides stained by hematoxylin and eosin (for eosinophils) or AB/PAS (for goblet and total epithelial cells) were examined with a light microscope (AX80, Olympus, Tokyo, Japan) connected to a digital camera (HC-300, Fuji Film, Tokyo, Japan) and an image capture software (Photograb-300, Fuji Film). Images of the intrapulmonary airway epithelium (largest airway in the specimen) were recorded from 5 consecutive high-power fields at $\times 400$. Image analysis was performed on a Power Macintosh G3 computer (Apple Computer, Cupertino, Calif.) using photo processing software (Photoshop 4.0J, Adobe Systems, San Jose, Calif.) and the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). Eosinophils in subepithelial area (within the limits of 0.1 mm in depth under subepithelial basement membrane) were counted. Data are expressed as the number of cells per millimeter subepithelial basement membrane.

The morphometric analysis of airway fibrosis was performed by a method described previously with some modification [13]. In brief, by using recorded images of the intrapulmonary airway stained by elastica-Masson's trichrome, we measured a basement membrane perimeter (Pbm) and the external perimeter (Pex) of area stained blue. On the assumption that both Pbm and Pex are circular, the area of collagen deposition (Acd) under basement membrane is given by $(Pex)^2/4\pi - (Pbm)^2/4\pi$. The mean $\pm$ SD of Pbm measured were 4.64 ± 0.40 , 4.71 ± 0.42 , 4.60 ± 0.34 and 4.66 ± 0.39 ($\times 10^{-1}$ mm; $n = 7-8$) for pretreatment, negative control, positive control and prolonged exposure groups, respectively. There are no significant differences between the groups ($p = 0.98$).

Assessment of Splenic Mononuclear Cell Proliferation

The spleens of mice were removed aseptically, and a single-cell suspension was obtained using a stainless steel mesh. Splenic mononuclear cells (SMNC) were obtained by a density-gradient method using Lympholyte-M (Cedarlane Lab., Ontario, Canada). The cells were suspended in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco), 100 U/ml penicillin G, 100 μ g/ml streptomycin, 50 mM 2-mercaptoethanol (Sigma Chemical Co.), and 10 mM HEPES (Sigma Chemical Co.). Cells were seeded (2.5×10^6 /ml) in 96-well flat-bottomed plates. For stimulation, 1 mg/ml OVA or 1 μ g/ml concanavalin A (Con A, Vector Labs, Burlingame, Calif.) or PBS was added to the cultures. SMNC were cultured at 37°C in a humidified incubator at 5% CO₂ atmosphere for 56 h. ³H-

thymidine (0.5 μ Ci/well) was added to the wells 8 h before harvesting, and incorporation of ³H-thymidine was measured with a liquid scintillation counter.

Measurement of Cytokines

The media of SMNC cultured as described above were collected at 48 h and stored at -80°C until use. Interleukin-2 (IL-2), IL-4, IL-5, IL-10, IL-12 (p70) and IFN- γ ELISA kits were purchased from Endogen (Boston, Mass.), and IL-13 and transforming growth factor- β 1 (TGF- β 1) were from R&D Systems (Minneapolis, Minn.). The concentrations of these cytokines were measured according to the procedures recommended by the manufacturer. TGF- β 1 were measured according to the procedures recommended by the manufacturer. In brief, cells were cultured with 10% FCS medium. After growth to confluence, the medium was then changed to serum-free medium supplemented with 0.02% crystalline BSA for over 12 h and cultured in medium containing 0.02% crystalline BSA for 24 h until collection. For activating the latent form of TGF- β , supernatants were treated with 10 mM HCl for 10 min at room temperature, followed by neutralization with 10 mM NaOH. The detection limits were 1.5 pg/ml for IL-13, 3 pg/ml for IL-2, 5 pg/ml for IL-4, IL-5 and IL-12, 7 pg/ml for TGF- β 1, 12 pg/ml for IL-10, and 15 pg/ml for IFN- γ .

Statistical Analysis

Data are expressed as mean $\pm$ SEM unless otherwise stated. Variables were analyzed using the Kruskal-Wallis test; the Mann-Whitney U test was used for comparisons between groups. $p < 0.05$ was accepted as statistically significant.

Results

Effects of Prolonged Antigen Exposure on AHR and Inflammatory Cells in BALF

In accordance with previously reported findings [11, 12], mice in the pretreatment stage developed a marked antigen-induced AHR to MCh. The positive control mice showed the same degree of AHR as the pretreatment mice (fig. 2). Prolonged exposure mice showed a significantly lower AHR than the positive control and pretreatment mice, and AHR in the prolonged exposure group was the same as AHR in the negative control group. Penh (enhanced pause) before exposure to aerosolized MCh (baseline Penh) did not differ significantly between the positive control group and the prolonged exposure group (0.41 ± 0.02 vs. 0.46 ± 0.02 , respectively).

Since the pretreatment mice were sensitized 3 times and challenged for 6 days, severe BALF eosinophilia was observed ($13.1 \pm 1.8 \times 10^5$ /ml) (fig. 3). Seven weeks later, decreased eosinophilia was observed in the positive control group ($17.2 \pm 3.6 \times 10^4$ /ml). However, mice treated with aerosolized antigen (prolonged exposure group) showed a significant decrease in eosinophils in BALF ($2.7 \pm 0.3 \times 10^4$ /ml) when compared to PBS-

Fig. 2. Effect on AHR. AHR to MCh was assessed by barometric whole-body plethysmography (see Materials and Methods). Antigen-induced AHR was observed during the pretreatment stage. In prolonged exposure group mice, AHR was suppressed to the level observed in negative control mice. Control treatment group mice (positive control group) showed increased AHR at the level of the pretreatment stage. Data are expressed as mean $\pm$ SEM of 8–10 mice per group. Baseline Penh after saline challenge in each mouse was considered as 100%. * $p < 0.005$; ** $p < 0.01$ vs. positive control group.

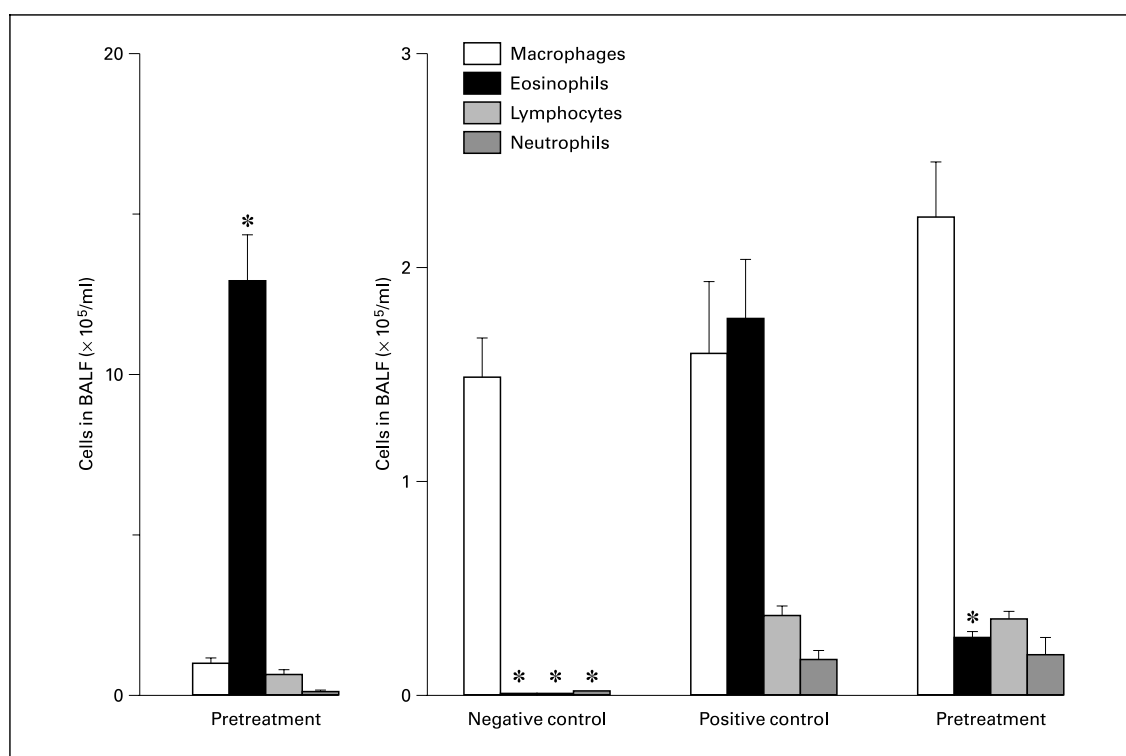
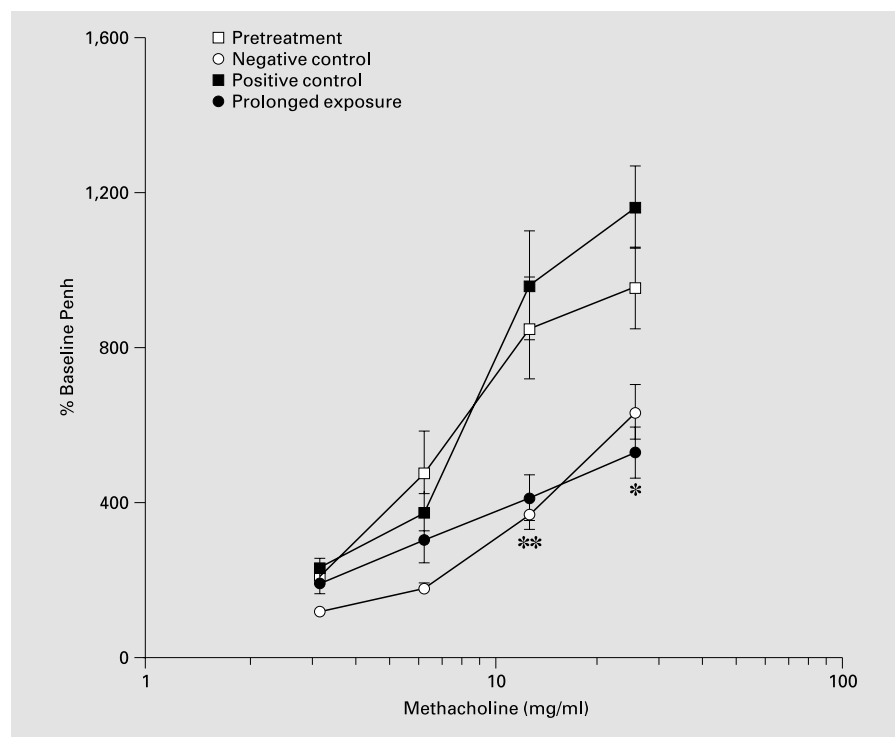


Fig. 3. Analysis of cells in BALF in pretreatment stage, negative control group, positive control group, and prolonged exposure group mice. After treatment periods, OVA challenge induced eosinophilia in BALF of positive control group mice, whereas there were much fewer eosinophils in the prolonged exposure group mice. Results are expressed as mean $\pm$ SEM of 6–9 mice. * $p < 0.005$ vs. positive control group.

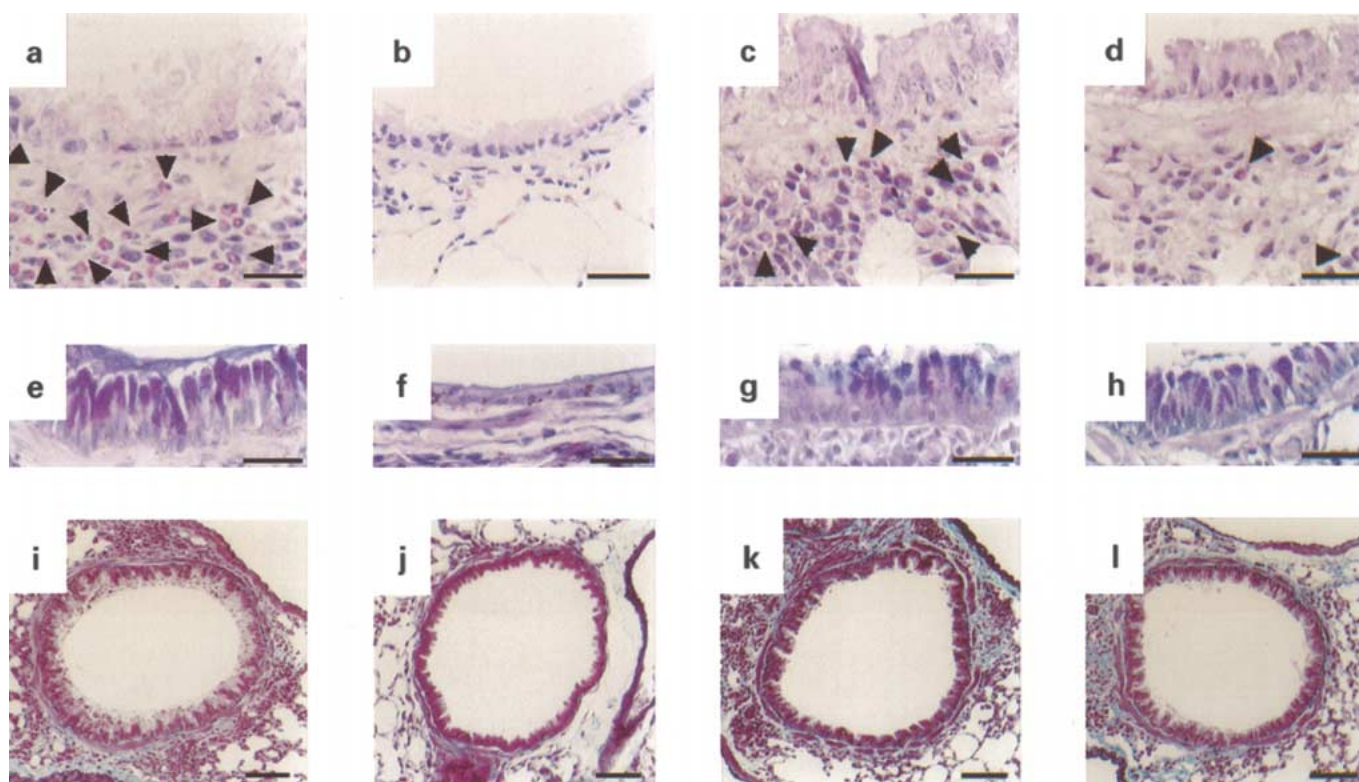


Fig. 4. Effects on histological changes in lung. Representative photos of the lungs from pretreatment (**a, e, i**), negative control (**b, f, j**), positive control (**c, g, k**) and prolonged exposure groups (**d, h, l**). Lung sections were stained with hematoxylin and eosin (**a–d**, bars = 50 μ m), AB/PAS (**e–h**, bars = 50 μ m), elastica-Masson's trichrome (**i–l**, bars = 100 μ m). Arrowheads show eosinophils.

Table 1. Serum OVA-specific antibody levels

Group	Ig isotype, U/ml	
	OVA-specific IgE	OVA-specific IgG2a
Pretreatment	568 $\pm$ 120	0 $\pm$ 0*
Negative control	0 $\pm$ 0*	0 $\pm$ 0*
Positive control	185 $\pm$ 72	1,548 $\pm$ 439
Prolonged exposure	6 $\pm$ 3*	2,170 $\pm$ 823

Serum levels of OVA-specific IgE and IgG2a were measured by ELISA as described in Materials and Methods, and results are shown as mean $\pm$ SEM of 6–9 mice. * $p < 0.005$ vs. positive control group.

treated mice (positive control group, $p < 0.005$). Prolonged exposure resulted in specific reduction of eosinophils without any significant effects on the other inflammatory cells in BALF.

Effect on OVA-Specific Antibody Levels

Mice in the pretreatment stage showed high OVA-specific IgE titers and undetectable levels of OVA-specific IgG2a (table 1). Chronic exposure to PBS or OVA resulted in decreased IgE and increased IgG2a levels. However, aerosolized OVA (prolonged exposure group) induced a significantly greater decrease in the level of OVA-specific IgE than did PBS (positive control group). There was, however, no significant difference in OVA-specific IgG2a titers between the two groups.

Histological Changes and Effect on Mucus Production

Mice in the pretreatment stage showed a marked subepithelial eosinophil-dominant cell infiltration (fig. 4a). However, the cell infiltration was less in both the positive control (fig. 4c) and prolonged exposure groups (fig. 4d). When compared to the positive control group, the prolonged exposure group had a significantly reduced number of subepithelial eosinophils, although the number in

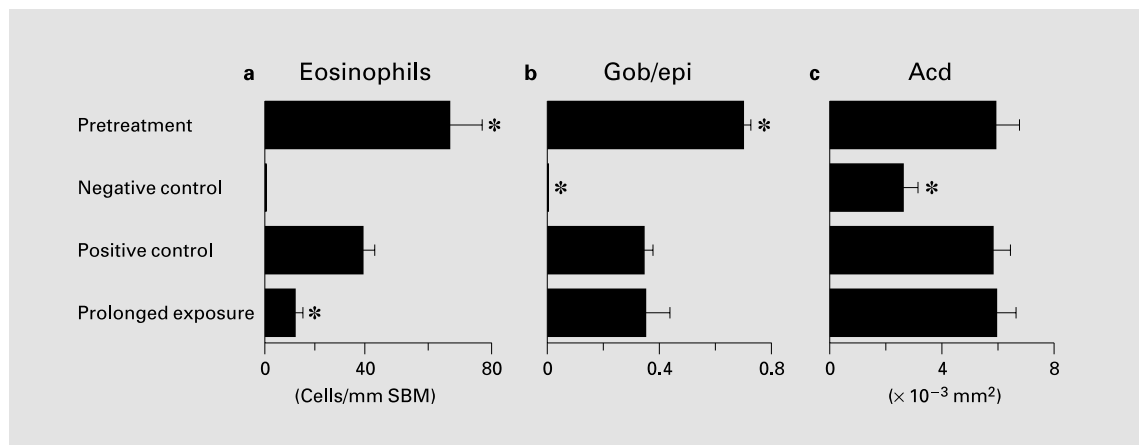


Fig. 5. Morphometric analysis of bronchial inflammation and airway remodeling. The subepithelial eosinophils (**a**), the goblet cell hyperplasia (**b**), and Acd of airway wall (**c**) are shown. Data are expressed as the number of cells per millimeter subepithelial basement membrane (SBM), the goblet cells/epithelial cells ratio (Gob/epi) or Acd (mm^2) (**a**, **b** $n = 6$; **c** $n = 7$ – 8 , mean $\pm$ SEM). * $p < 0.005$ vs. positive control group.

Table 2. Cytokine profiles in BALF

Group	IL-4 pg/ml	IL-5 pg/ml	IL-13 pg/ml	IFN- γ pg/ml	IL-12 pg/ml
Negative control	<5	<5**	<1.5***	<15*	40 $\pm$ 6
Positive control	<5	84 $\pm$ 18	29 $\pm$ 7	102 $\pm$ 26	77 $\pm$ 18
Prolonged exposure	<5	7 $\pm$ 2***	4 $\pm$ 1**	47 $\pm$ 16	559 $\pm$ 101***

Cytokine levels were measured by ELISA as described in Materials and Methods. Results are shown as mean $\pm$ SEM of 6–10 mice. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$ vs. positive control group.

both of these groups was much less than the number in the pretreatment group (fig. 5a).

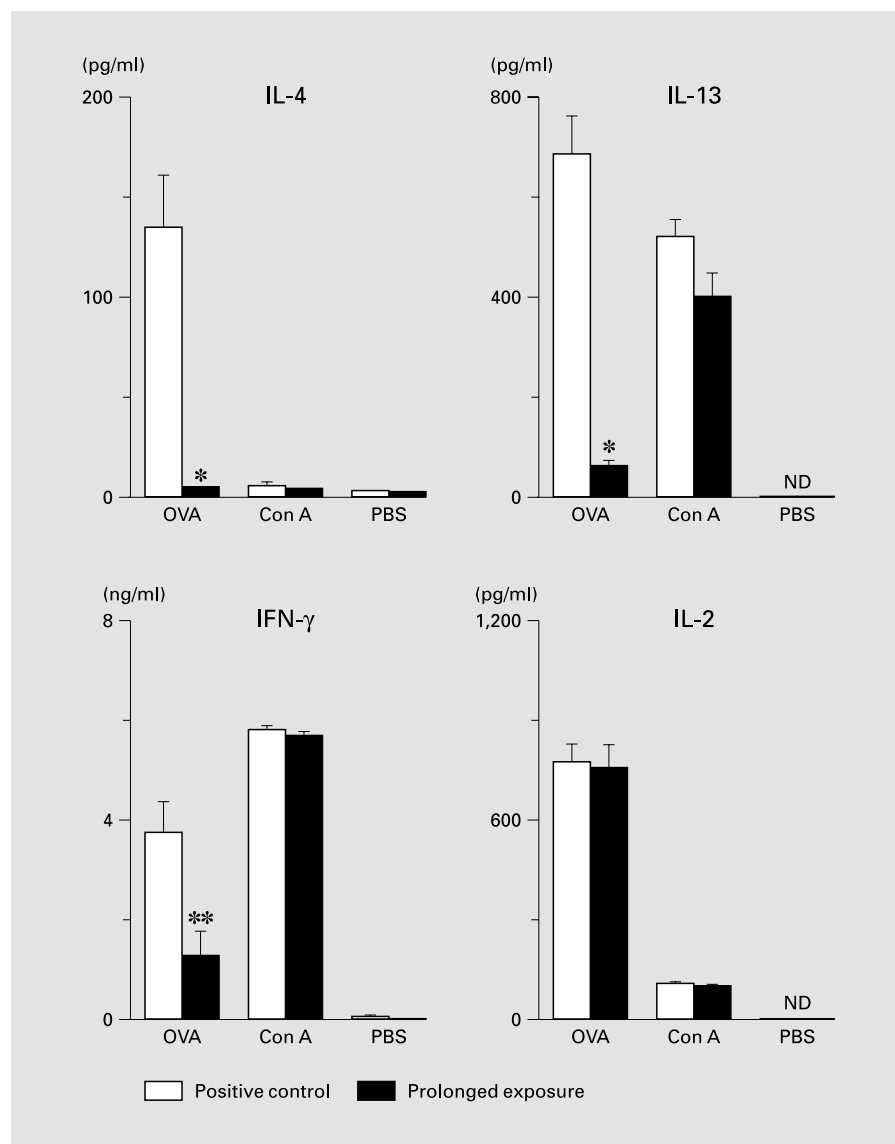
AB/PAS-staining mucus and cells (goblet cells) were observed in the groups except in the negative control group, and were most prominent in the pretreatment group (fig. 4e). That is, there was no significant difference in the number of goblet cells between the positive control group and the prolonged exposure group (fig. 5b). Subepithelial collagen fibers around the airways were observed in all but the negative control group (fig. 4i–l). There were no significant differences in the Acd among pretreatment, positive control and prolonged exposure groups (fig. 5c). Thickening of the basement membrane was not observed in any group.

Effect on the Concentration of Cytokines in BALF

To understand the underlying mechanism(s) for the phenotypic differences between the prolonged exposure and positive control mice, we further analyzed BALF and spleen cells of the two treatment groups.

The BALF cytokine levels are shown in table 2. IL-4 was not detected in either group. Levels of other Th2 cytokines, IL-5 and IL-13 were significantly lower in the prolonged exposure group than in the positive control group. IL-10 levels did not differ between groups (13.1 ± 2.6 vs. 14.8 ± 2.7 , prolonged exposure vs. positive control group; $p = 0.75$). The IFN- γ level was lower in the prolonged exposure group than in the positive control group, but the difference was not significant ($p = 0.06$). The IL-12 (p70) level in the prolonged exposure group, however, was almost 8 times higher than that in the positive control group.

Fig. 6. Profiles of cytokine production of SMNC from positive control group mice and prolonged exposure group mice. SMNC were stimulated with 1 mg/ml OVA (n = 10) or 1 µg/ml of Con A (n = 4). OVA-stimulated SMNC from the prolonged exposure group showed significantly lower IL-4, IL-13 and IFN-γ production than OVA-stimulated SMNC from the positive control group (* p < 0.0005; ** p < 0.005). OVA-stimulated IL-2 production did not differ significantly. Con A-stimulated SMNC from the prolonged exposure group produced cytokines at the same levels as SMNC from the positive control group. Data are expressed as mean ± SEM.



Proliferation and Cytokine Production of SMNC in Response to OVA Stimulation

SMNC from both groups showed good proliferative responses to OVA and Con A (OVA: 5.2 ± 0.4 vs. $5.0 \pm 0.2 \times 10^4$ cpm, Con A: 6.2 ± 0.8 vs. $7.7 \pm 0.5 \times 10^4$ cpm, positive control group vs. prolonged exposure group), and there were no significant differences between the groups. The production of cytokines from OVA-stimulated SMNC is shown in figure 6. Th2 cytokines, IL-4 and IL-13 were of significantly higher levels in the positive control than in the prolonged exposure group. The levels of IL-5 and IL-10 (not shown in fig. 6) were also higher in the positive control than in the prolonged expo-

sure group (IL-5: 313 ± 43 vs. 32 ± 6 , $p < 0.0005$; IL-10: $1,047 \pm 136$ vs. 174 ± 42 , $p < 0.0005$; positive control group vs. prolonged exposure group). The Th1 cytokine IFN-γ level was also significantly higher in the positive control group than it was in the prolonged exposure group, whereas the IL-2 level did not differ between groups ($p = 0.62$).

At the same time, cytokines in the culture supernatant of Con A-stimulated SMNC were measured. IL-4, IL-13, IFN-γ and IL-2 levels did not differ between the prolonged exposure and positive control groups. IL-12 was not detectable in either group under either stimulation condition (data not shown).

TGF- β Levels in BALF and Culture Supernatant of OVA-Stimulated SMNC

TGF- β was detected in BALF but not in culture supernatant (data not shown). The level in BALF was significantly higher in the positive control group than it was in the prolonged exposure group (124 ± 2 and 26 ± 6 pg/ml, respectively, $p < 0.05$).

Discussion

We demonstrated that prolonged antigen exposure can markedly inhibit airway eosinophilia, antigen-induced AHR and OVA-specific IgE response in a mouse allergic asthma model. Goblet cell hyperplasia of the airway epithelium and airway fibrosis were not inhibited by treatment. These effects were accompanied by suppression of both local (BALF) and systemic (splenic) Th2 cytokine production as well as markedly increased levels of IL-12 in BALF. The present results suggest that prolonged antigen exposure potentially inhibits AHR and airway eosinophilia but that it has no effect on airway remodeling in the murine asthma model.

Amelioration of airway eosinophilia, IgE response and antigen-induced AHR observed in our prolonged exposure group could be explained by the suppression of Th2 cytokines. Recent studies have established a role for IL-5 in tissue eosinophilia [14, 15]. IL-4 and IL-13 play key roles in IgE production, mucus hypersecretion and AHR [16–18]. IL-13 is known to induce goblet cell hyperplasia and airway fibrosis in mice [18]. However, investigation into the mechanism of airway remodeling has just begun, and the process is not fully understood [19]. The dissociation between AHR and airway remodeling is an interesting finding, but the mechanism is not known at present.

As mentioned earlier, exposing naive mice to aerosolized OVA induces IgE-specific tolerance, which is transferable to naive mice via T cells from treated mice [6–8]. The same mechanism may be responsible for the inhibition observed in the present study. The responsible cells may be IFN- γ -producing T cells in respiratory tract lymph nodes and splenocytes. Administration of IL-10 is reported to suppress Th2 cytokines and eosinophilia in asthma model mice [20]. Recent studies disclosed a new immunoregulatory T cell population, TGF- β -secreting T cells (designated 'Th3' cells). Th3 cells are reported to be induced by low-dose oral and nasal immunotherapy [21, 22]. In the present study, neither IFN- γ , IL-10 nor TGF- β increased in BALF or in the culture supernatant of OVA-stimulated splenocytes. The proliferative response of

SMNC to OVA and IL-2 secretion from OVA-stimulated SMNC were not inhibited by a prolonged exposure to antigen aerosols. Therefore, splenic OVA-reactive T cells were not anergic and it was unlikely that they were skewed simply from Th2 to Th1.

The striking feature of our prolonged exposure group mice was the high BALF IL-12 levels, which were 8 times higher than in the PBS-treated or negative control mice. Local or systemic IL-12 administration in the mouse asthma model is known to inhibit not only the development of AHR and airway eosinophilia but also the production of Th2 cytokines [23–25]. Therefore, increased IL-12 production in the lungs observed in the present study may have largely contributed to the inhibited murine asthma pathophysiology. It should be mentioned that IL-12 is known as an inducer of IFN- γ [26]. In our study, however, IFN- γ did not increase in spite of a high level of IL-12 in BALF. Although the reason for this discrepancy cannot be explained, more profound regulatory mechanisms may be involved. In addition, there seems to be a relationship between high IL-12 levels in BALF and persisted airway remodeling, although it has not been proved at present.

Atopy is one of the strongest predisposing factors in the development of asthma. However, it has been found in a high percentage (30–50%) of the general population. Therefore, atopy is frequently found in individuals without clinical asthma. In other words, continuous exposure to environmental allergens induces airway inflammation in certain but not all atopic individuals. The present results may be relevant for atopic individuals without asthma. Chronic exposure to allergens in such individuals may result in a downregulation of the process for the induction and persistence of the asthmatic airway inflammation. Anyway, the mechanism for attenuating the asthmatic phenotype, as observed in the present study, would be important for understanding the process for the development of atopic asthma.

In conclusion, prolonged antigen exposure in a murine asthma model induces marked suppression of AHR, airway eosinophilia, and IgE response to antigen in association with an inhibition of Th2 cytokines and augmentation of lung IL-12 production.

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