

Mouse Model of Airway Remodeling

Strain Differences

Kazuhiko Shinagawa and Masami Kojima

Pharmacology Lab, Kissei Pharmaceutical Co. Ltd., Nagano, Japan

We found that continuous eosinophilic inflammation after repeated antigen instillation into the nose was observed only in A/J mice, not in three other strains. Histologic analysis of tissues from A/J mice revealed features typical of airway remodeling, i.e., airway wall thickening and increased collagen depositions were observed after 12 weeks' antigen exposure. Persistent airway hyperresponsiveness (AHR) was observed in chronically antigen-exposed A/J mice. Eosinophilic inflammation, collagen deposition, and airway wall thickening were all less marked in BALB/c mice than in A/J mice, and no AHR was observed in the former strain. In C57BL/6 and C3H/HeJ mice, eosinophilic inflammation, airway wall thickening, and AHR were not observed at all, although slightly increased collagen deposition was observed. Thus, we found that these changes were strain-dependent. On the other hand, in A/J mice *inhalational* antigen challenge after ovalbumin/alum immunization led only to a transient increase in eosinophils and to less airway wall thickening, indicating the importance of the protocol used. Use of A/J mice and giving antigen by instillation via the nose is to be recommended for studies of the mechanisms underlying asthma. In particular, useful qualitative and quantitative information relating to the structural and histologic changes in the lungs may be obtainable using this model.

Keywords: asthma; eosinophil; airway hyperresponsiveness; A/J mouse

Asthma is characterized by chronic inflammation of the airway walls with infiltration by eosinophils and lymphocytes (1, 2). In addition, airway morphology in asthma not only displays the characteristics expected of an acute inflammatory process but also structural changes (2–17). The term “airway remodeling” is now widely used to refer to the development of specific structural changes in the airway wall in asthma. Postmortem studies have demonstrated that the pathologic features of acute and chronic airway inflammation include epithelial shedding (2–4), thickening of the basement membrane (lamina reticularis) (5–7), an increase in blood vessel cross-sectional area (7–10), airway smooth-muscle hyperplasia and hypertrophy (6–8, 11–13), mucous gland and goblet cell hyperplasia (6–8, 13–16), and increased collagen deposition (17). The thickening around the airways has been postulated to be a critical mechanism in the maintenance and intractability of chronic asthma and in the deterioration due to this disease (18). Contrariwise, it has been argued that airway remodeling may actually represent a protective mechanism against excessive airway narrowing (19). In fact, it is very difficult to demonstrate definitive correlations between subepithelial thickness or airway wall smooth-muscle mass and airway function in humans. There-

fore, suitable animal models are needed to enable us properly to address this question.

New therapeutic strategies to prevent airway remodeling in asthma are urgently needed, and the importance of developing and characterizing animal models has recently been stressed (20). However, simple, reproducible murine models of the chronic changes that occur in the airways are lacking because most mouse models are relatively short term (21). In fact, chronically challenged mice develop antigen tolerance, a condition characterized by a progressive loss of the initial airway eosinophilia (*see* Figure 1 in this paper). It has also been reported that eosinophilic inflammation and airway hyperresponsiveness (AHR) is lost during chronic challenge (22). Therefore, the lungs in such animals are devoid of the chronic inflammatory and epithelial changes that typify human asthma (21). Temelkovski and co-workers addressed these limitations by developing a novel protocol that involves chronic inhalational challenge of ovalbumin (OVA)-sensitized mice by controlled exposure to low-mass concentrations of aerosolized antigen (23, 24). They succeeded in eliciting chronic eosinophilic inflammation, a condition in which mice exhibit abnormalities of the airway epithelium as well as AHR. However, it is difficult to give controlled aerosolized antigen, and it is difficult to repeat this type of experiment exactly in another laboratory. For these reasons, simple murine asthma models that show chronic eosinophilia and AHR are needed.

Recently, several reports have been published on the genetic factors associated with asthma in human studies (25, 26). In addition, bronchial responsiveness has been shown to differ among inbred mouse strains (27). On the basis of these reports, we speculated that mice possessing a genetic asthma-prone background would exist. To try to identify such mice, we screened four mouse strains by giving antigen into the nose without using adjuvant and determined whether asthmatic responses were evoked. We found that there were marked strain differences in airway inflammation as well as in the AHR and histologic changes induced by this maneuver.

METHODS

Animals

All experimental procedures conformed to international standards of animal welfare and were approved by the Laboratory Animal Committee of Kissei Pharmaceutical Co. Ltd.; they also conformed to current Japanese law. Male A/J, BALB/c, C57BL/6, and C3H/HeJ mice (SPF; 5 weeks old) purchased from SLC Japan Inc. (Shizuoka, Japan) were maintained under a 12-hour light–dark cycle with free access to water and standard laboratory food until the day of the experiment.

Induction of Eosinophilic Inflammation by Immunization with OVA/Alum

Mice were immunized and boosted by injection with 10 µg of OVA (grade V; Sigma, St. Louis, MO) adsorbed onto Al(OH)₃ gel (Serva Electrophoresis GmbH, Heidelberg, Germany) as previously described (28, 29, Figure 1A). Then, mice were challenged 5 days per week with an aerosol of OVA (10 mg/ml). To obtain bronchoalveolar lavage (BAL) samples, mice were anesthetized with urethane (25%, intraperitoneally) and the lungs lavaged with phosphate-buffered saline (PBS). BAL fluid

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Correspondence and requests for reprints should be addressed to Kazuhiko Shinagawa, Pharmacology Lab, Kissei Pharmaceutical Co. Ltd., 4365-1, Kashiwabara, Hotaka, Minamiazumi, Nagano 399-8304, Japan. E-mail: kazuhiko_shinagawa@pharm.kissei.co.jp

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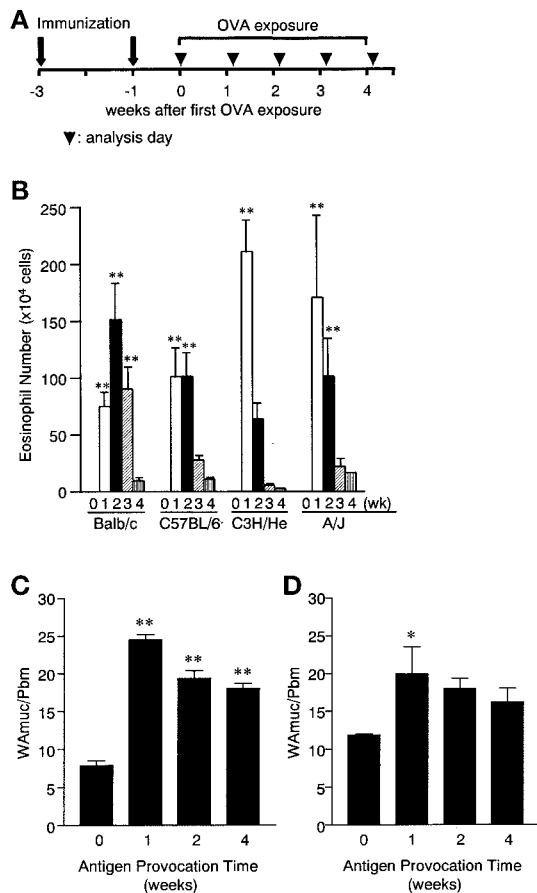


Figure 1. (A) Protocols for immunization with ovalbumin (OVA)/alum and subsequent OVA exposure by inhalation 5 days per week for 4 weeks. Mice were killed 24 hours after the last OVA challenge. (B) Bronchoalveolar lavage (BAL) cells were counted and dispersed onto microscope slides using a cytospin. Cytospin preparations were stained to obtain a differential leukocyte count. Eosinophils counts in BAL samples obtained from four strains of mice. Means $\pm$ SEM; $n = 6$ mice for each time point. * p Value less than 0.05, ** p value less than 0.01 versus nonsensitized mice. C and D showed the effects of repeated OVA exposure on airway wall thickness in bronchi in A/J (C) and BALB/c (D) mice. Values for cross-sectional areas of mucous layer were obtained according to the methods described by Bai and coworkers (31) (see METHODS). Means $\pm$ SEM; $n = 4$ mice for each time point. * p Value less than 0.05, ** p value less than 0.01 versus untreated mice (0 weeks).

on cytospin slides was stained with Diff-Quick (Baxter Healthcare, McGaw Park, IL).

Induction of Eosinophilic Inflammation by Intranasal Administration of OVA

Mice were placed in a small box and anesthetized with inhaled enflurane (Abbott, IL) supplied by a small-animal anesthesia machine (type K-1; Igarashi Ika Kogyo, Tokyo, Japan). Anesthetized mice were instilled with OVA (1 mg/ml) intranasally using a micropipette (50 μ l). Administration of OVA was performed 3 days per week for various periods. Twenty-four hours after the last administration of antigen, the mice were anesthetized with urethane and the lungs lavaged four times with sterile PBS. BAL fluid on cytospin slides was fixed and stained with Diff-Quick. Differential cell counts were obtained under the light microscope. Total BAL cells were counted using a hemocytometer, and supernatants were collected and analyzed for ELISA.

Measurement of Total IgE in Serum

Total serum IgE was measured using a capture ELISA according to the method described previously (30).

Histologic Analysis

Twenty-four hours after the last antigen challenge, mice were anesthetized with urethane and killed for the preparation of lung sections. Histologic analysis was performed using a BX50 microscope (Olympus, Tokyo, Japan) attached to a MacSCOPE image analysis system (Mitani, Fukui, Japan). For each treatment group, 15 to 20 stained lung sections from each of six mice were analyzed as reported previously (31).

Hydroxyproline Assay

Total lung collagen was determined by analysis of hydroxyproline as reported by Nagatani and coworkers (32).

Assessment of Airway Responsiveness in Anesthetized and Conscious Mice

In anesthetized mice, AHR was measured 24 hours after the last intranasal antigen challenge according to the method of Ohta and coworkers (33). Briefly, anesthetized mice were tracheostomized and placed on a Harvard ventilator, then placed inside whole-body plethysmographs (Buxco Electronics, Inc., Troy, NY) to measure airway resistance (Raw). Increasing doses of methacholine (5–1,280 mg/kg) were administered intravenously.

In conscious mice, airway responsiveness was measured by recording the Penh values (using whole-body plethysmography [Buxco Electronics]) obtained in response to inhaled methacholine.

Cytokine Assay

Cytokine levels in supernatants from BAL fluids were measured using ELISA kits (for interleukin (IL)-4, IL-5, and IFN- γ [Endogen, Woburn, MA], transforming growth factor- β (TGF- β) [Pharmingen, San Diego, CA], and IL-13 [R&D Systems Inc., Minneapolis, MN]).

Peribronchial Lymph Node Cells: Isolation and Culture Conditions

Peribronchial lymph node (PBLN) cells were isolated from the PBLNs by mechanical disruption and then filtered through nylon mesh (Cell Strainer; Becton Dickinson, Franklin Lakes, NJ). Single-cell suspensions of PBLN cells were cultured in RPMI medium (supplemented with 10 μ g/ml gentamicin, 50 μ M 2-mercaptoethanol, and 10% fetal calf serum) and stimulated with 10 μ g/ml OVA for 96 hours.

Statistical Analysis

Data are given as mean $\pm$ SEM. An analysis of variance with Dunnett's test for multiple comparisons was used to determine the statistical significance of differences from the values obtained for nonsensitized animals after OVA-challenges had been delivered for various periods. A two-way analysis of variance with Tukey's multiple range test was used to determine the statistical significance of differences among strains after OVA challenges had been delivered for various periods. p Values less than 0.05 was considered statistically significant.

RESULTS

Decline in Initial Increases in Eosinophils and Lymphocytes during Chronic Exposure to OVA Aerosol in Mice Immunized with OVA/Alum

We sensitized and challenged four strains of mice by the most commonly used method. Mice immunized with OVA/alum and then chronically challenged 5 days per week with an OVA aerosol showed significant increases in the numbers of BAL cells at 1 to 2 weeks (compared with nonsensitized mice). In particular, eosinophils showed marked increases in all four strains (Figure 1). The number of eosinophils peaked at 1 week in C3H/HeJ and A/J mice, at 1 to 2 weeks in C57BL/6 mice, and at 2 weeks in BALB/c mice (Figure 1). However, in all four strains the number then declined despite continued OVA exposure throughout Weeks 3 and 4. In all four strains, the number of lymphocytes showed a transient increase, peaking in Weeks 1 to 2 before declining in Weeks 3 and 4 (data not shown).

To investigate the extent of airway remodeling in these mice, the cross-sectional areas of the mucous and smooth muscle layers were obtained by the method of Bai and coworkers (31) using a computer graphics analysis of slides. In the thickness of the airway smooth-muscle layer, no significant changes were observed in any strain of antigen-exposed mice. The thickness of the mucous cell layer was increased by a 1-week antigen exposure in all strains, but it was not increased further by repeated antigen exposure over a period of 4 weeks.

Numbers of Inflammatory Cells in BAL and Serum IgE Levels after Chronic Antigen Instillation into the Nose

Because the common immunization method failed to induce persistent eosinophilia, as shown in Figure 1, we investigated another method (nasal instillation). Except where otherwise noted, all the results described below were obtained using this second method. We found that strong and persistent eosinophilic inflammation was induced in A/J mice by instillation of OVA solution into the nose on 3 days per week without systemic immunization (Figure 2). That is to say, eosinophils were increased at 4 weeks, and their number remained steady from Week 4 to Week 12 (Figure 2B). The number of eosinophils was two times that seen after inhalational challenge using OVA. This increase in eosinophils was concomitant with increases in lymphocytes and macrophages (Figure 2C). The total number of BAL cells from A/J mice was increased by 4 weeks OVA exposure, and the elevation persisted up to Week 12. In contrast, BALB/c, C3H/HeJ, and C57BL/6 strains showed much smaller, or nonexistent, increases in inflammatory cells at 4 weeks despite being challenged using the same protocol (Figure 2B), and the counts were not increased even by more prolonged antigen exposure.

The serum IgE level was very low in untreated mice of all four strains, but it was increased by OVA exposure for 4 to 12 weeks in each strain (Figure 2D). In A/J mice, the IgE level was higher at 8 and 12 weeks than at 0 weeks. In BALB/c mice, it was increased at 4 and 8 weeks, but at 12 weeks it had declined

markedly toward the Week 0 level. The IgE levels achieved in C57BL/6 mice were lower than those seen in A/J and BALB/c mice. Therefore, IgE levels were increased roughly in parallel with the eosinophil numbers in BAL in these three strains (compare Figure 2D with Figure 2B). On the other hand, no such association was observed between eosinophilia and IgE levels in C3H/HeJ mice: IgE levels were, or tended to be, increased at 4 to 12 weeks, but few eosinophils were found in BAL in this strain.

Chronically OVA-challenged Mice Show Histologic Changes, Such as Increased Airway Wall Mass and Collagen Deposition

We next made a histologic analysis of lung sections taken from the four strains of mice after OVA exposure by intranasal instillation (Figures 3 and 4). A/J mice, OVA-exposed for 12 weeks showed a marked increase in inflammatory cells in the lung mucosal and submucosal areas (Figures 3B and 3D). In addition, eosinophil recruitment into the lamina propria of the trachea was observed, and some eosinophils were found in the airway lumen (Figure 3D, *arrowheads*). To try to quantify tissue inflammation, the total number of inflammatory cells present in a 40,000 μm^2 area of lung tissue was counted (Figure 3E). The number of inflammatory cells was significantly greater in the tissue of 4 weeks OVA-exposed A/J mice than in that of nonexposed mice (0 weeks), and it then remained steady from Weeks 8 to 12.

Many of the epithelial cells seemed to be enlarged due to the accumulation within their cytoplasm of homogeneous-looking material that stained positively with periodic acid–Schiff, indicating that most of these cells were probably mucous cells such as goblet cells (Figures 4B and 4D). The smooth-muscle layer in the small airways from allergen-exposed A/J mice was thicker (Figure 4B) than that in untreated mice (Figure 4A). Masson trichrome staining of lung tissue from A/J mice demonstrated a markedly increased deposition of collagen in the lamina propria (Figure 4F), which was not observed in the airways of untreated

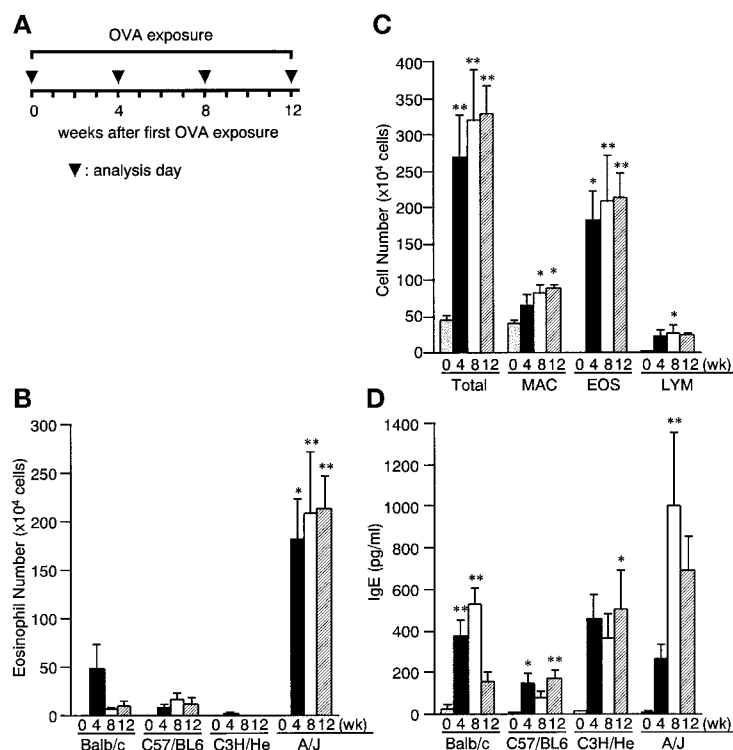


Figure 2. (A) Timeline showing the protocol used for ovalbumin (OVA) exposure (by instillation of OVA solution into the nose 3 days per week) in four strains of mice without systemic immunization. (B) Eosinophil counts in bronchoalveolar lavage (BAL) samples obtained from four strains of mice after various periods of OVA exposure. (C) Shows a breakdown of the total counts obtained in A/J mice. Mice were killed 24 hours after the last OVA challenge. BAL cells were counted and dispersed onto microscope slides using a cytopsin. Cytopsin preparations were stained to obtain a differential leukocyte count. Means $\pm$ SEM; $n = 6$ mice for each timepoint. * p Value less than 0.05, ** p value less than 0.01 versus untreated mice. (D) Shows serum IgE levels in four strains of OVA-exposed mice. Serum IgE was measured using a capture ELISA, as described in METHODS. Means $\pm$ SEM; $n = 6$ mice per group for each timepoint. * p Value less than 0.05, ** p value less than 0.01 versus OVA-untreated mice.

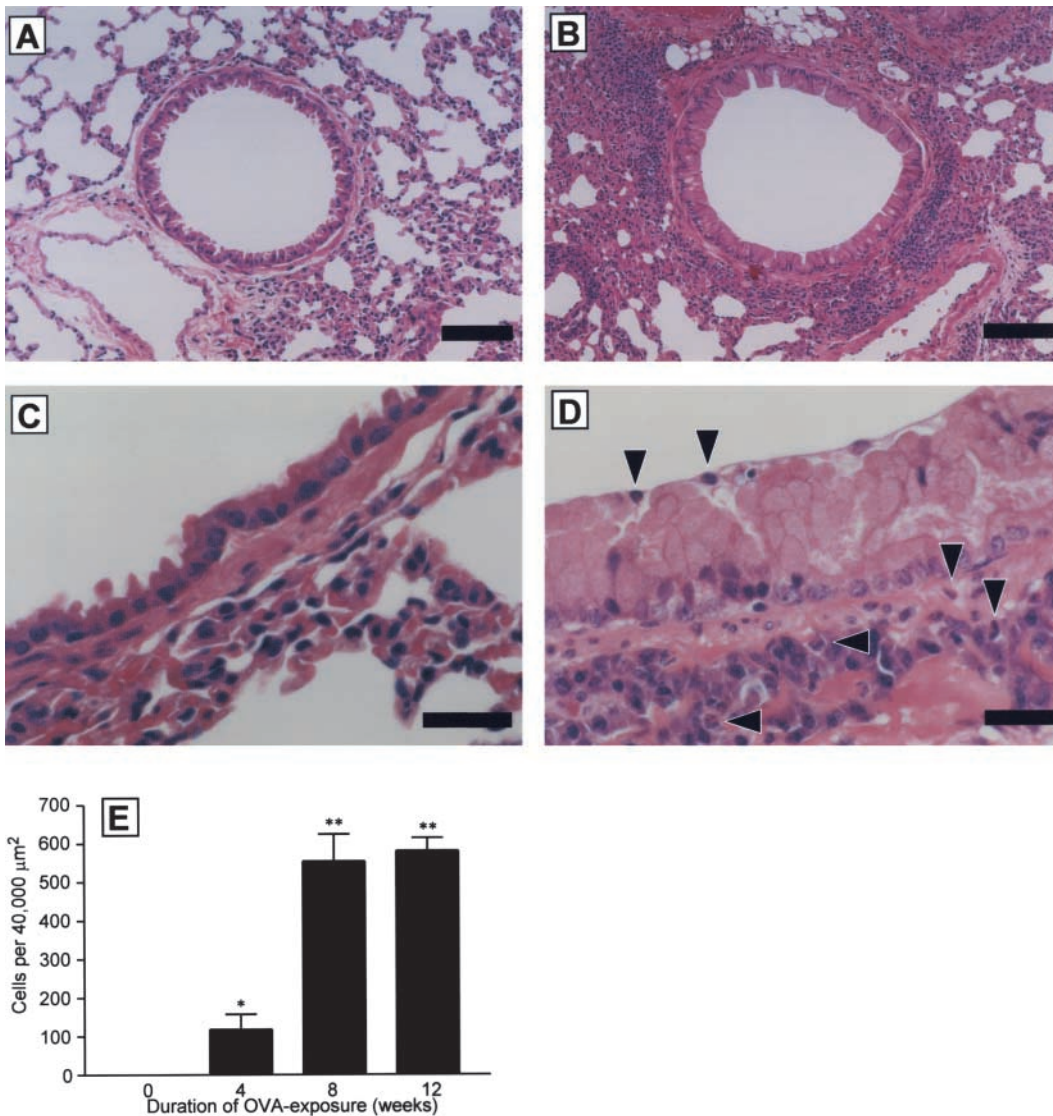


Figure 3. H&E staining of bronchial sections from A/J mice. The photomicrographs are representative of lungs removed from ovalbumin (OVA)-challenged (B, D) and untreated (A, C) mice. In OVA-exposed mice, increases in inflammatory cells (arrowheads) were apparent around the airways. Bar = 400 μm (A, B) and bar = 100 μm (C, D). E shows the peribronchial inflammatory cell count in the lungs of allergen-challenged mice (within a 40,000 μm^2 area). *p Value less than 0.05, **p value less than 0.01 versus OVA-untreated mice.

mice (Figure 4E). These histologic changes observed in A/J mice were not observed in C57BL/6 or C3H/HeJ mice (data not shown). In BALB/c mice, increases were observed in the smooth-muscle layer and the mucous layer, although the changes were less marked than in A/J mice (data not shown).

Measurements of Hydroxyproline Content of Lungs in Chronically OVA-exposed Mice

To quantify the deposition of collagen, the hydroxyproline content of the lungs was measured in both untreated and 4- to 12-weeks OVA-challenged mice. In A/J mice, the hydroxyproline content was progressively increased by prolonged antigen exposure (Figure 5), the increases being by 1.09, 1.50, and 1.80 times at 4, 8, and 12 weeks. In the other three strains, although the hydroxyproline content increased as the time of antigen exposure was increased, the increase was less marked than in A/J mice. These results indicate that collagen was deposited in lung tissue by repeated antigen exposure and support the results obtained using Masson trichrome staining.

Measurements of Airway Wall Area in Chronically OVA-exposed Mice

To investigate the extent of airway remodeling, the cross-sectional areas of the mucous and smooth muscle layers in both untreated

and 4- to 12-weeks OVA-challenged mice were obtained by the method of Bai and coworkers (31) using a computer graphics analysis of slides. In A/J mice, the thickness of the mucous cell layer was increased from the early stages of the sensitization, the mucous membrane being roughly three times thicker in 4-weeks OVA-challenged mice than in untreated mice (Figure 6B). This increase was maintained from Week 4 to Week 12. On the other hand, compared with that in the normal control mice, the smooth-muscle layer showed a progressive thickening, being increased by 1.49, 1.63, and 2.25 times at 4, 8, and 12 weeks, respectively (Figure 6A). In BALB/c mice, the cross-sectional areas of the mucous cell and smooth muscle cell layers were both increased significantly at 8 and 12 weeks (Figures 6C and 6D). In contrast, in C57BL/6 and C3H/HeJ mice there were no significant increases in either layer (Figures 6E and 6H).

Change in Airway Hyperresponsiveness in Chronically OVA-challenged Mice

Anesthetized A/J mice were more sensitive to methacholine than the other strains (note different scales on ordinates and abscissae in Figure 7A). In untreated A/J mice, intravenous injection of methacholine elicited a dose-dependent increase in Raw over the dose range 5 to 80 mg/kg (Figure 7A). The responses to

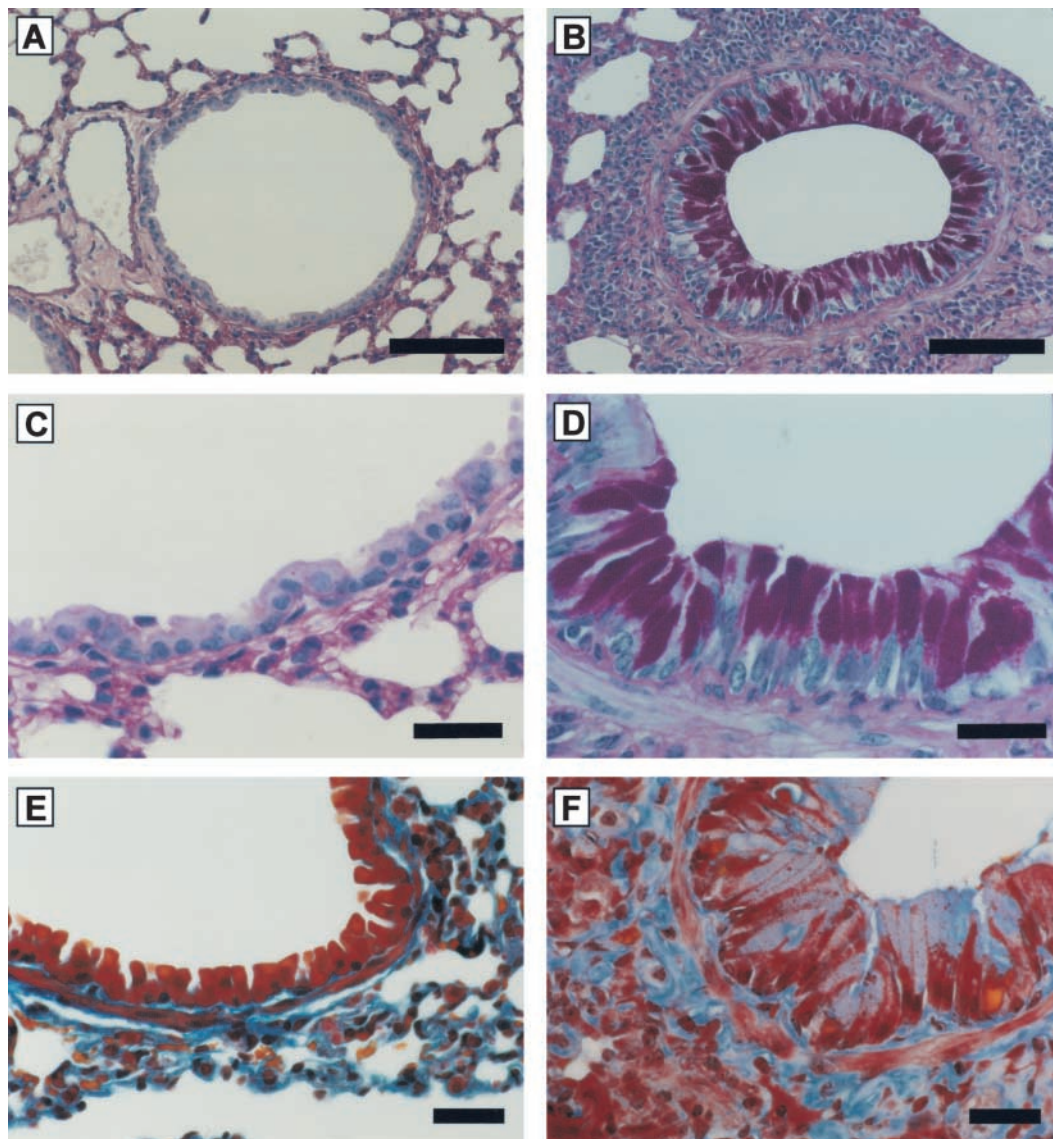


Figure 4. Periodic acid-Schiff (A–D) and Masson trichrome (E, F) staining of whole-lung sections from A/J mice. The photomicrographs are representative of lungs removed from ovalbumin (OVA)-challenged (B, D, F) and untreated (A, C, E) mice. In OVA-exposed A/J mice, goblet cell hyperplasia and markedly increased collagen deposition was apparent around the airways. Bar = 400 μ m (A, B) and bar = 100 μ m (C–F).

methacholine shown by OVA-exposed A/J mice were characteristic of AHR, with a left-shifted curve and an increased maximal reactivity. By comparison with those in the untreated A/J mice, the Raw values obtained after methacholine administration were (1) already greater at 4 weeks of OVA exposure (at 20–80 mg/kg methacholine), (2) also greater at 8- and 12-weeks OVA exposure (at 20–80 mg/kg and 10–80 mg/kg methacholine, re-

spectively). Significant changes in Raw were not observed in BALB/c, C57BL/6, or C3H/HeJ mice after 4- to 12-weeks OVA exposure.

To investigate lung function in chronically antigen-exposed conscious mice, we measured the Penh value, which is an indicator of Raw. In A/J mice, the Penh value was increased by methacholine exposure, and this effect was significantly enhanced in the chronically (8- to 12-week) antigen-exposed mice (Figure 7E), a result much the same as that obtained by measurements of Raw (Figure 7A).

Cytokine Levels in BAL after Repeated Antigen exposure

We found that the levels of cytokines in BAL fluids differed among the four strains at any given time point. By comparison with the levels in untreated mice (Week 0), Th2 cytokines (IL-4, IL-5, and IL-13) were increased in OVA-exposed A/J mice at 4 to 8 weeks, although these levels had declined again at 12 weeks (Table 1). In particular, the IL-13 level was very high compared with the level in untreated mice. Furthermore, this value was higher than those obtained for the other Th2 cytokines (1.7 times that for IL-4, 4.7 times that for IL-5 at 4 weeks). On the other hand, the level of the Th1 cytokine IFN- γ tended to

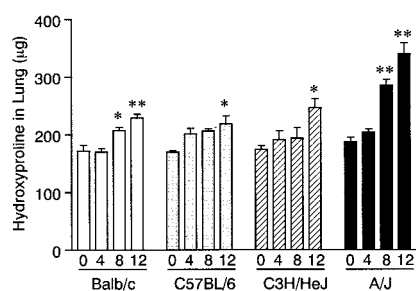


Figure 5. Levels of hydroxyproline (total collagen) in lung tissue of four strains of mice. Hydroxyproline in lung tissue of mice exposed to intranasal ovalbumin (OVA) was measured and expressed in micrograms per lung. Data represent the mean $\pm$ SEM; n = 4 to 6 mice for each time point. *p Value less than 0.05, **p value less than 0.01 versus untreated mice (0 weeks).

SEM; n = 4 to 6 mice for each time point. *p Value less than 0.05, **p value less than 0.01 versus untreated mice (0 weeks).

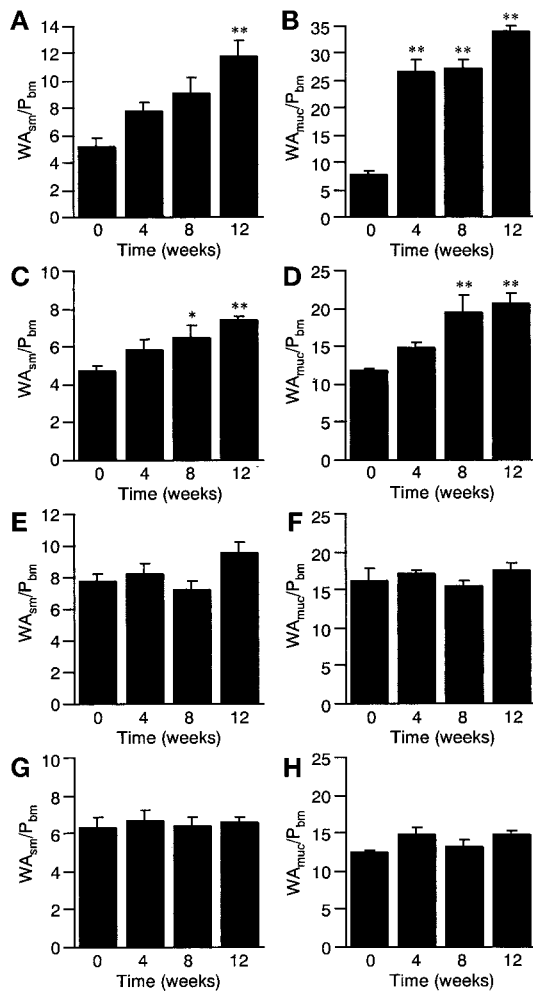


Figure 6. Effects of repeated ovalbumin (OVA) exposure on airway wall thickness in bronchi in A/J (A, B), BALB/c (C, D), C57BL/6 (E, F), and C3H/HeJ (G, H) mice. Values for cross-sectional areas of smooth-muscle layer (A, C, E, G) and mucous layer (B, D, F, H) were obtained according to the methods described by Bai and coworkers (31) (see METHODS). Means $\pm$ SEM; $n = 6$ mice for each time point. Note different ordinate scales among the various panels. * p Value less than 0.05, ** p value less than 0.01 versus untreated mice (0 weeks).

be lower in antigen-exposed A/J mice than in untreated mice, indicating that the Th1/Th2 balance was biased toward Th2. Interestingly, the TGF- β level was very high in 8- to 12-weeks OVA-exposed A/J mice, even though it was not detectable in nonexposed mice. In contrast to the situation in A/J mice, in BALB/c the Th2 cytokine levels were, or tended to be, increased by 4-weeks antigen exposure, but did not continue to increase during prolonged exposure (up to 12 weeks). In particular, the IL-13 level in BALB/c mice was not increased (in contrast to the situation in A/J mice). In BALB/c mice, IFN- γ tended to be at lower levels than in untreated mice, although the change was very small. In BALB/c mice, TGF- β levels tended to be increased by OVA exposure, but less so than in A/J mice (at 12 weeks). In C3H/HeJ and C57BL/6 mice, although the levels of Th2 cytokines were not greatly changed, the IFN- γ levels were decreased by antigen exposure.

Cytokine Production from PBLN Cells Prepared from Repeatedly Antigen-exposed Mice

We next examined cytokine production from PBLN cells prepared from 4 to 12 weeks OVA-exposed mice. PBLN cells were

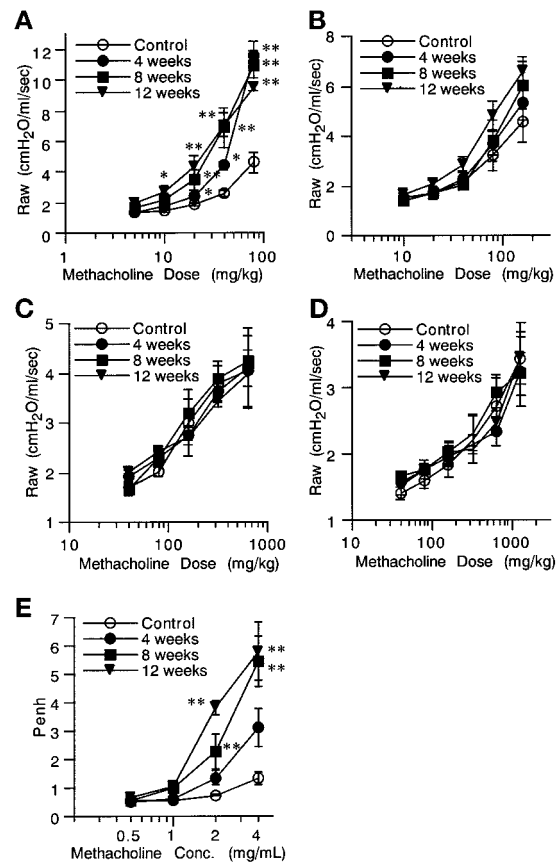


Figure 7. Airway hyperresponsiveness (AHR) (as assessed by changes in airway resistance (Raw) or Penh) induced by increasing concentrations of methacholine (Mch) in A/J (A, E), BALB/c (B), C57BL/6 (C), and C3H/HeJ (D) mice. A–D show values for Raw obtained from anesthetized mice intravenously injected with Mch. (E) shows Penh value obtained from conscious mice exposed to aerosolized Mch. Means $\pm$ SEM; $n = 4$ to 6 mice for each time point. * p Value less than 0.05, ** p value less than 0.01 versus ovalbumin (OVA)-untreated mice (0 weeks).

stimulated with OVA (10 μ g/ml) for 96 hours (Figure 8). PBLN cells obtained from A/J mice produced large quantities of Th2 cytokines and IFN- γ . Interestingly, IL-13 production from PBLN cells was very large (~ 130 times that recorded for IL-4, and 5 times that for IL-5) (note different ordinate scales in Figure 8). PBLN cells from BALB/c mice produced Th2 cytokines at 4 weeks, but not at later time points. On the other hand, PBLN cells obtained from C57BL/6 and C3H/HeJ mice did not produce any Th2 cytokines. PBLN cells from nontreated mice (Week 0) produced no cytokines in any of the four strains (data not shown).

DISCUSSION

The results obtained in this experiment indicate that delivering antigen by instillation into the nose in A/J mice is useful for evaluating airway remodeling, the three main reasons being as follows. (1) Persistent eosinophilic inflammation was observed only in A/J mice after prolonged antigen exposure (up to 12 weeks). Previous reports indicated that the number of eosinophils decreases as the period of antigen provocation is increased (21), and this was the result we obtained when we used an OVA aerosol after OVA/alum immunization (Figure 1). Hence, the common method, involving an inhalational antigen challenge after OVA/alum immunization to mice, is not suitable for establishing

TABLE 1. CYTOKINE LEVELS IN BRONCHOALVEOLAR LAVAGE OBTAINED FROM FOUR STRAINS OF MICE (NONSENSITIZED OR OVALBUMIN-EXPOSED)

Strain	Time (wk)	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-13 (pg/ml)	IFN- γ (pg/ml)	TGF- β (pg/ml)
A/J	0	3.4 $\pm$ 3.4	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	81.6 $\pm$ 3.0 ^{§¶}	0.0 $\pm$ 0.0
	4	84.4 $\pm$ 16.8 ^{†§**}	30.4 $\pm$ 16.1	141.7 $\pm$ 45.9 ^{†§**}	52.3 $\pm$ 4.8 ^{†§**}	408.0 $\pm$ 152.8
	8	78.9 $\pm$ 23.5 ^{†,§,}	88.8 $\pm$ 3.1*	118.2 $\pm$ 25.6 ^{†§**}	15.2 $\pm$ 3.5 [†]	3,648.8 $\pm$ 377.6 ^{†§, **}
	12	45.0 $\pm$ 4.9	13.0 $\pm$ 8.0	51.8 $\pm$ 9.2	17.5 $\pm$ 2.4 [†]	2157 $\pm$ 608.2 ^{†§, **}
BALB/c	0	2.0 $\pm$ 1.8	79.0 $\pm$ 16.7	0.0 $\pm$ 0.0	13.9 $\pm$ 2.5	0.0 $\pm$ 0.0
	4	60.6 $\pm$ 10.9 ^{†§}	72.5 $\pm$ 5.1	9.8 $\pm$ 2.2	8.8 $\pm$ 1.5	757.4 $\pm$ 269.8
	8	27.5 $\pm$ 14.5	66.8 $\pm$ 19.0	1.7 $\pm$ 1.1	6.4 $\pm$ 0.9	470.7 $\pm$ 181.7
	12	0.2 $\pm$ 0.2	39.3 $\pm$ 18.1	9.1 $\pm$ 5.7	8.2 $\pm$ 1.8	740.5 $\pm$ 218.8
C57BL/6	0	7.8 $\pm$ 3.8	71.5 $\pm$ 23.3	0.0 $\pm$ 0.0	21.8 $\pm$ 2.1	122.0 $\pm$ 67.7
	4	1.5 $\pm$ 1.1 [§]	7.2 $\pm$ 7.2	1.0 $\pm$ 0.6	7.2 $\pm$ 0.9 [†]	373.2 $\pm$ 127.4
	8	13.9 $\pm$ 3.7	37.6 $\pm$ 15.4	4.9 $\pm$ 2.8	4.7 $\pm$ 0.40 [†]	256.2 $\pm$ 85.0
	12	4.0 $\pm$ 2.9	24.6 $\pm$ 15.1	3.1 $\pm$ 2.0	7.2 $\pm$ 1.8 [†]	429 $\pm$ 208.8
C3H/HeJ	0	35.0 $\pm$ 9.7	85.1 $\pm$ 4.3	0.0 $\pm$ 0.0	21.3 $\pm$ 2.6	0.0 $\pm$ 0.0
	4	18.1 $\pm$ 14.2	11.6 $\pm$ 11.6*	0.5 $\pm$ 0.5	7.8 $\pm$ 1.1 [†]	73.1 $\pm$ 19.5
	8	27.0 $\pm$ 10.0	44.2 $\pm$ 18.4	0.0 $\pm$ 0.0	4.2 $\pm$ 1.0 [†]	120.5 $\pm$ 19.6
	12	3.8 $\pm$ 3.5	58.5 $\pm$ 14.8	0.2 $\pm$ 0.2	6.1 $\pm$ 1.0 [†]	308.5 $\pm$ 165.5

Definition of abbreviations: BAL = bronchoalveolar lavage; IL = interleukin; OVA = ovalbumin; TGF- β = transforming growth factor- β .

Cytokine levels (IL-4, IL-5, IL-13, IFN- γ , and TGF- β levels) in BAL fluids were measured using ELISA, as described in METHODS. BAL samples were obtained from nonsensitized mice (0 weeks) and from mice OVA-exposed for 4 or 12 weeks.

Means $\pm$ SEM; n = 6 mice per group.

*p < 0.05 vs. nonsensitized mice.

†p < 0.01 vs. nonsensitized mice.

‡p < 0.05 vs. BALB/c mice.

§p < 0.01 vs. BALB/c mice.

¶p < 0.01 vs. C57BL/6 mice.

||p < 0.05 vs. C3H/HeJ mice.

**p < 0.01 vs. C3H/HeJ mice.

persistent eosinophilic inflammation. We therefore examined another method and found that strong and persistent eosinophilic inflammation was induced in A/J mice when the antigen was instilled into the nose rather than delivered as an inhalational challenge after OVA/alum immunization. Such persistent eosinophilic inflammation was not induced in the three other strains, and was thus strain-dependent. In addition, the method of antigen provocation is also important because A/J mice given an OVA-aerosol for 2 weeks after OVA/alum immunization showed only

a transient increase in eosinophils. (2) Marked histologic changes were observed in A/J mice as the antigen challenge period was increased. In our histologic investigation, airway smooth-muscle and mucous cell layers were revealed to be thickened in A/J mice given OVA by nasal instillation. Furthermore, our hydroxyproline analysis provided evidence of an increase in collagen deposition in the lung. These histologic changes were more severe in A/J mice than in the other three strains. (3) A persistence of the AHR elicited by methacholine was observed only in A/J

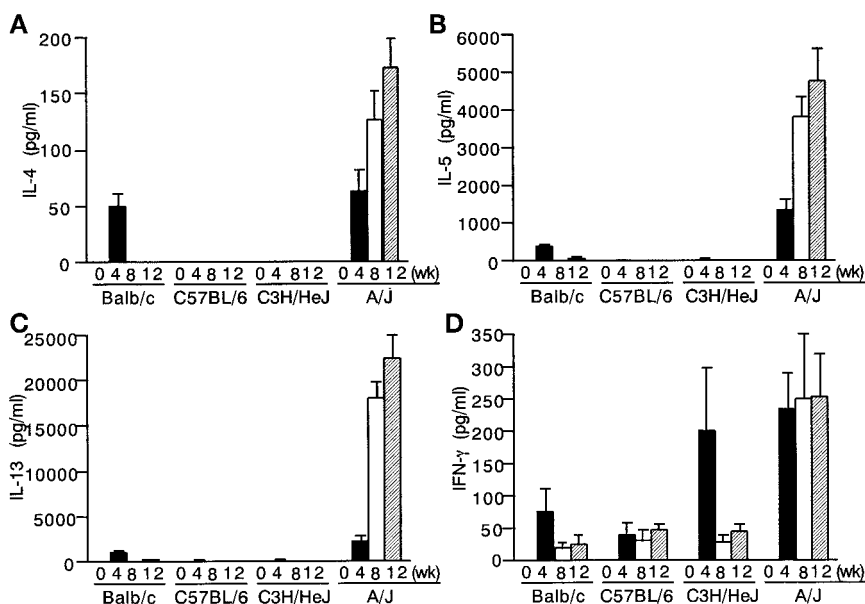


Figure 8. Cytokine production from peribronchial lymph node (PBLN) cells obtained from repeatedly antigen-exposed mice. PBLN cells were stimulated with ovalbumin (OVA) (10 μ g/ml) for 96 hours. Each panel shows means $\pm$ SEM of three cultures obtained from the PBLN cells obtained from each of five to six mice of each strain. Data are expressed as pg/ml/ 2×10^5 cells. *p Value less than 0.05, **p value less than 0.01 versus OVA-untreated mice (0 weeks).

mice during repeated antigen exposure for up to 12 weeks. To judge from these results, the intranasally antigen-exposed A/J-mouse model is a good one for the study of the airway remodeling that occurs in asthma.

In BALB/c mice, antigen tolerance in terms of BAL eosinophils was observed as the time of antigen exposure was increased, although such antigen tolerance in BAL eosinophils was not observed in A/J mice. The same result was observed for Th2 cytokine levels in BAL. Therefore, we speculate that T cell tolerance occurred in BALB/c mice but not in A/J mice when both were challenged chronically. However, cytokine levels in BAL may not accurately reflect the cytokine production from T cells because cytokines may be produced by other cells in the lung or they might be consumed during the induction of allergic inflammation. Therefore, we prepared PBLN cells and stimulated them with antigen to investigate cytokine production from T cells. It was clear that Th2 cytokines were increasingly produced by PBLN cells from A/J mice as the time of antigen exposure was increased. On the other hand, Th2 cytokines were not produced in BALB/c mice after 8- or 12-weeks antigen exposure, although small amounts of Th2 cytokines were produced at 4 weeks. Therefore, T cell tolerance occurred in BALB/c mice, but not in A/J mice. We cannot explain this difference between A/J mice and BALB/c mice in our results, but others have indicated that TGF- β and IL-10 are important for the induction of tolerance (34, 35). However, we could not detect any TGF- β production from OVA-stimulated PBLN cells in any of the four strains of mice at any time point over the range of 4 to 12 weeks (data not shown). Therefore, Th3 and/or Tr1 cells, which are important for tolerance by virtue of their secretion of TGF- β and IL-10, may not have been involved in the induction of tolerance in our experiment. However, a more detailed examination is needed because our failure to detect TGF- β may have been a consequence of the consumption of this cytokine. Interestingly, Swirski and coworkers recently reported the importance of the granulocyte-macrophage colony-stimulating factor in chronically antigen-exposed BALB/c mice (36). They showed that in antigen-tolerant mice, which showed reduced airway eosinophilia after repeated antigen exposure for up to 4 weeks, eosinophilia was fully restored by the delivery of the recombinant granulocyte-macrophage colony-stimulating factor. We measured the granulocyte-macrophage colony-stimulating factor in BAL from BALB/c mice and found that this cytokine was increased in mice exposed to antigen for 4 weeks, but then decreased to the levels seen in untreated mice after repeated antigen exposure, although it remained increased in chronically (8- to 12-weeks) antigen-exposed A/J mice (data not shown). On this basis, the granulocyte-macrophage colony-stimulating factor seems to be important for tolerance, but further study will be needed. Indeed, we intend to carry out a more detailed comparison between these two strains at some time in the future in the hope that this might provide an explanation for the difference in tolerance they exhibit.

It is clear that both the strain used and the protocol employed for antigen sensitization/challenge affect the development of pulmonary allergic disease (which is characterized by eosinophilic inflammation, AHR, and airway remodeling). Temelkovski and coworkers succeeded in developing a new model in BALB/c mice that is characterized by persistent eosinophilic inflammation, lesions of airway mucus and collagen production, and AHR (23, 24). A feature of their method is to expose the animals to low-mass concentrations of aerosolized antigen in a strictly controlled way. However, they noted that no airway lesion or AHR were elicited in C57BL/6 mice when they were exposed to low levels of aerosolized antigen. This emphasizes the importance of the strain used (in addition to the importance

of the protocol employed for antigen sensitization/challenge). Wills-Karp and colleagues recently reported that inbred strains of mice are genetically predisposed to be either susceptible or resistant to the bronchoconstrictor effects of acetylcholine under inflammatory and noninflammatory conditions (37). Although their protocol for inducing an inflammatory response involved only one challenge to the trachea, their results are consistent with ours. That is to say, their examination of susceptibility to AHR after an antigen challenge gave a rank order of A/J = AKR/J > BALB/c > C57BL/6 > C3H/HeJ, which is similar to the order obtained by us. In addition, they found the number of eosinophils in BAL to be increased more in A/J mice than in the other strains, which is also very similar to our finding. Furthermore, Yasue and coworkers reported that A/J mice are high responders, whereas C57BL/6 and C3H/HeJ mice are low responders to other antigens (Der-f 1 and 2) (38). Taking these reports and our results together suggest that it is best to use A/J mice to study allergic asthma. However, Brewer and coworkers reported conflicting results in a study of 12 strains of mice (39). In their study, A/J mice were resistant to eosinophilic inflammation and AHR. We speculate that this may reflect differences in the method of airway antigen exposure. Thus, as mentioned previously, strain is important for pulmonary allergic inflammation, but the protocol used for antigen sensitization/challenge is also very important if the allergic reaction is to be sustained.

In conclusion, our study emphasizes that genetic background (mouse strain) is important for the establishment of the persistent eosinophilic inflammation that is closely related to the development of airway remodeling. At present, the most suitable murine asthma model for investigating airway remodeling would seem to be the intranasally antigen-instilled A/J-mouse model because marked histologic changes occur, and these are useful for quantifying airway wall thickening. Furthermore, this model is also suitable for the study of cytokine levels and eosinophilic inflammation because these do not decline on prolonged antigen exposure in A/J mice, although they do in the other strains examined.

Conflict of Interest Statement: K.S. has no declared conflict of interest; M.K. has no declared conflict of interest.

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