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Altered allergen-induced eosinophil trafficking and physiological dysfunction in airways with preexisting virus-induced injury

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Sorkness RL, Herricks KM, Szakaly RJ, Lemanske RF Jr, Rosenthal LA. Altered allergen-induced eosinophil trafficking and physiological dysfunction in airways with preexisting virus-induced injury. *Am J Physiol Lung Cell Mol Physiol* 292: L85–L91, 2007. First published August 11, 2006; doi:10.1152/ajplung.00234.2006.—Although both asthmatics and allergic rhinitics develop an acute inflammatory response to lower airway allergen challenge, only asthmatics experience airway obstruction resulting from chronic environmental allergen exposure. Hypothesizing that asthmatic airways have an altered response to chronic allergic inflammation, we compared the effects of repeated low-level exposures to inhaled *Alternaria* extract in sensitized rats with preexisting chronic postbronchiolitis airway dysfunction versus sensitized controls with normal airways. Measurements of air space (bronchoalveolar lavage) inflammatory cells, airway goblet cells, airway wall collagen, airway wall eosinophils, airway alveolar attachments, and pulmonary physiology were conducted after six weekly exposures to aerosolized saline or *Alternaria* extract. Postbronchiolitis rats, but not those starting with normal airways, had persistent increases in airway wall eosinophils, goblet cell hyperplasia in small airways, and loss of lung elastic recoil after repeated exposure to aerosolized *Alternaria* extract. Despite having elevated airway wall eosinophils, the postbronchiolitis rats had no eosinophils in bronchoalveolar lavage at 5 days after the last allergen exposure, suggesting altered egression of tissue eosinophils into the air space. In conclusion, rats with preexisting airway pathology had altered eosinophil trafficking and allergen-induced changes in airway epithelium and lung mechanics that were absent in sensitized control rats that had normal airways before the allergen exposures.

postbronchiolitis sequelae; lung elastic recoil; rats; *Alternaria*

MOST PERSONS WITH ASTHMA have allergies, and there are strong epidemiologic associations between exposure to allergens and both the inception and the aggravation of asthma (15, 31, 36). Direct evidence for links between allergens and asthma include worsening of asthma during 4 wk of dust mite exposure mimicking the level found in bedrooms (1) and improvement of asthma during allergen avoidance (44). However, not all atopic persons who are exposed to allergens have an asthmatic phenotype, even though pulmonary allergen challenge in nonasthmatic allergic subjects elicits an acute eosinophilic inflammatory response that resembles that of asthmatic subjects (5, 22, 43). One possible mechanism explaining this may be that a persistent asthma phenotype develops as a consequence of usual environmental inflammatory stimuli interacting with airways made vulnerable by an initial inflammatory event occurring during a period of maturation of both the lung structure and the immune system (19).

We therefore hypothesized that asthmatic airways are more vulnerable than normal airways to chronic low-level allergen exposure, resulting in a more persistent inflammatory milieu that contributes to airway dysfunction. To address this hypothesis, we used the rat model of postbronchiolitis chronic airway dysfunction (39). In this model, rats are infected with parainfluenza virus early in life, and in the weeks after resolution of the bronchiolitis the animals develop chronic airway dysfunction that has several features in common with human asthma, including spontaneous airflow obstruction that improves with corticosteroid treatment, hyperresponsiveness to methacholine, peribronchiolar inflammatory infiltrates, and airway wall thickening with subepithelial collagen deposition (6, 24, 47, 49, 50). The chronicity of the postbronchiolitis airway pathology allowed us to investigate potential interactive effects between preexisting airway pathology and repeated low-level *Alternaria* allergen exposure, comparing rats with asthmalike airways versus control rats matched for age, sex, genetic background, allergen sensitization, and environment.

METHODS

Study design. Figure 1 illustrates the study design. A period of 8 wk between the viral inoculation and the first allergen challenge was selected so that the acute inflammation and repair associated with viral bronchiolitis would be resolved before the allergen sensitization and challenge procedures, so that the physiological changes associated with acute viral illness would be resolved (46), and so that the rats would be sufficiently grown to allow use of the same size of tracheal tube for baseline and ending physiology studies. The 6-wk length for repeated allergen exposures was selected based on previously published studies in rats that reported airway remodeling after 4–12 wk of allergen exposures repeated every 2–3 days (17, 21, 35). In contrast to previous studies, which were designed to obtain allergen-induced changes in rats starting with normal airways, our intent was to induce a submaximal response, using less frequent challenges and more dilute allergen concentrations, in order to detect a difference in sensitivity of the rats with preexisting airway abnormalities. The terminal studies were conducted 5 days after the final allergen challenge in order to focus on the persistent effects rather than the acute effects of the allergen challenge.

Animals and viral procedures. Inbred male Brown Norway rats (BN/SsNHsd substrain; Harlan, Indianapolis, IN) were purchased as 3- to 4-wk-old weanlings and housed in an accredited biosafety level 2 animal facility with HEPA-filtered air. All animal procedures conformed to the *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Wisconsin Animal Care and Use Committee. One-half of the rats were inoculated with aerosolized

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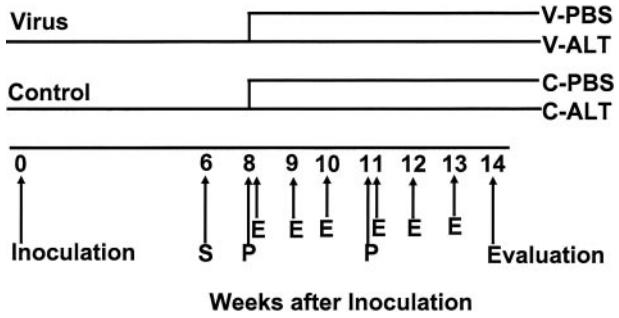


Fig. 1. Experimental design. After inoculation with Sendai virus (Virus) or sham inoculum (Control), rats were sensitized to *Alternaria* extract (S) and exposed to aerosolized PBS or *Alternaria* extract 6 times (E). Physiology measurements (P) were conducted at baseline and midpoint, in addition to the final evaluation [physiology, bronchoalveolar lavage (BAL), and lung histology] 5 days after the last aerosol exposure. V-PBS, virus inoculated-PBS challenged; V-Alt, virus inoculated-*Alternaria* extract (Alt) challenged; C-PBS, control inoculated-PBS challenged; C-Alt, control inoculated-Alt challenged.

parainfluenza type 1 (Sendai) virus to induce a viral bronchiolitis and postbronchiolitis chronic airway dysfunction, as described previously (49), and the other half were sham-inoculated controls. One of the sham-inoculated rats was found to have a pneumonitis unrelated to study procedures and was excluded from the protocol before aerosol challenges had commenced.

Allergen sensitization and challenges. Two weeks before the first allergen challenge, all the rats were sensitized to crude extract of *Alternaria alternata* (Alt) [extract 62% protein; obtained from the laboratory of Dr. Robert Bush (Department of Medicine, University of Wisconsin, Madison, WI); Ref. 4] with a single subcutaneous injection of extract (1 mg protein equivalent) along with adjuvant (Imject Alum, Pierce, Rockford, IL). Weekly exposures to aerosolized Alt or PBS were begun, using an Ultraneb 99 ultrasonic nebulizer (De Vilbiss, Somerset, PA) to deliver aerosol for 20 min into a box containing the rats (48). A 0.5% solution of Alt was used for the first challenge to initiate an inflammatory response, and the remaining Alt exposures used a 0.1% solution.

Physiology studies. Physiology studies were conducted a day before the first allergen exposure, before the fourth exposure (midpoint of the study), and 5 days after the sixth exposure. Pulmonary physiology was assessed in lightly anesthetized rats instrumented with an orotracheal tube, using a total body plethysmograph equipped with software-controlled valves to measure thoracic gas volume and forced expiratory volumes (FEVs) as described and characterized previously (49). The FEV in 0.2 s ($FEV_{0.2}$) and the forced vital capacity (FVC), each normalized to total lung capacity (TLC), were used as the primary indicators of airway function (49). The $FEV_{0.2}$ in a normal rat instrumented with an orotracheal cannula is ~84% FVC and thus is analogous to the FEV_1 in a young adult human (49). Quasi-static deflation pressure-volume curves were obtained during a slow deflation from TLC (30 cmH_2O); pressure at the airway opening (transrespiratory pressure) at 60%, 70%, and 80% TLC was used to compare lung elastic recoil among groups.

Assessment of airway inflammation and remodeling. Pulmonary inflammation and remodeling were assessed by obtaining cells from the air space via bronchoalveolar lavage (BAL) of the right lung and by quantitative histology from paraffin sections of the left lung (48). The total BAL leukocyte count was determined with a cell counter (Beckman Coulter, Hialeah, FL), and the differential cell count was obtained from 200 cells on a cytospin slide stained with eosin-methylene blue stain. Eosinophils were identified on cytospin slides by their red-stained granules and their characteristically shaped nuclei. Lung sections were stained for either collagen and mucin, with a modified trichrome stain with Alcian blue, or eosinophil major basic protein (MBP), with rabbit polyclonal anti-MBP (a gift from Dr. James Lee, Division of Pulmonary Medicine, Mayo Clinic, Scottsdale, AZ), peroxidase-conjugated secondary antibody, Vector VIP substrate (Vector Laboratories, Burlingame, CA) and methyl green counterstain (25). From each lung section digital images were obtained for 8–15 airways in cross section having short-to-long diameter ratio >0.5 and basement membrane perimeters of 500–3,000 μm. With SigmaScan Pro 5 software (SPSS Science, Chicago IL), basement membrane perimeter and airway wall area were measured (2), and the area within the airway wall stained for MBP or for collagen was measured with a color threshold. Goblet cells were identified by the Alcian blue mucin stain, and the number in each airway was recorded. The number of radial alveolar attachments to the airway wall was evaluated for each of the airways obtained from trichrome-stained sections by the method of Saetta et al. (40).

Data analysis. Data were analyzed with general linear ANOVA models for data conforming to parametric assumptions, and Kruskal-Wallis/Mann-Whitney procedures as nonparametric alternatives (SYSTAT version 11, SYSTAT Software, Richmond, CA). A repeated-measures model was used to compare changes in $FEV_{0.2}$ over time and to compare lung elastic recoil over the range of three lung volumes. For quantitative histology, airways were grouped by size in five categories of 500-μm increments of basement membrane perimeter between 500 and 3,000 μm. Airway wall MBP and collagen areas were normalized to basement membrane perimeter, log-transformed,

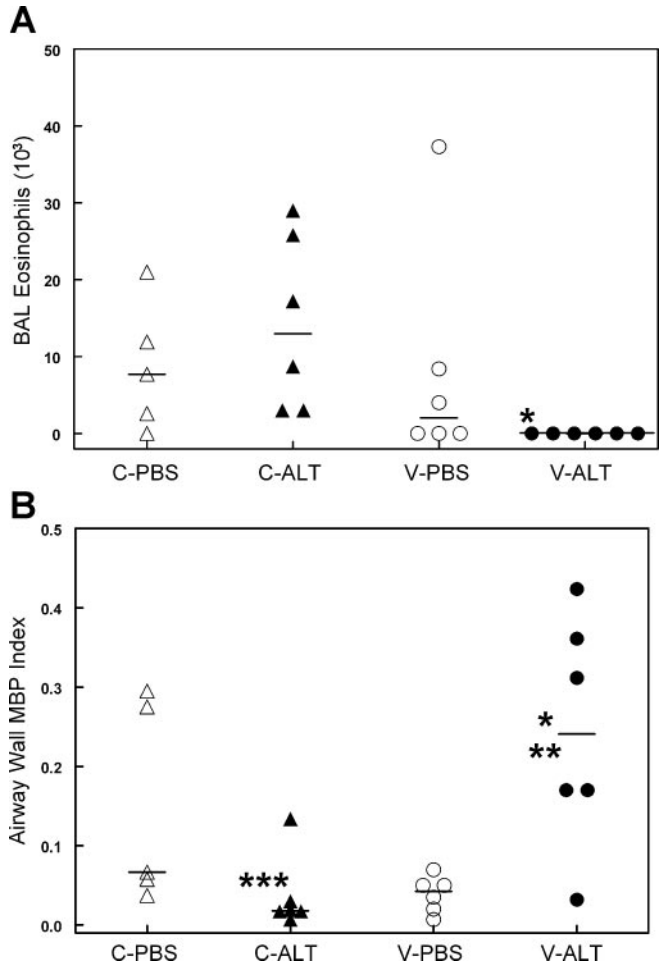


Fig. 2. Eosinophilic inflammation. *A*: no. of eosinophils recovered via right lung BAL in individual rats from each treatment group. *B*: airway wall major basic protein (MBP) staining in individual rats. Bars indicate group medians. *$P \leq 0.002$ vs. C-Alt; **$P < 0.0001$ vs. V-PBS; ***$P < 0.0001$ vs. C-PBS.

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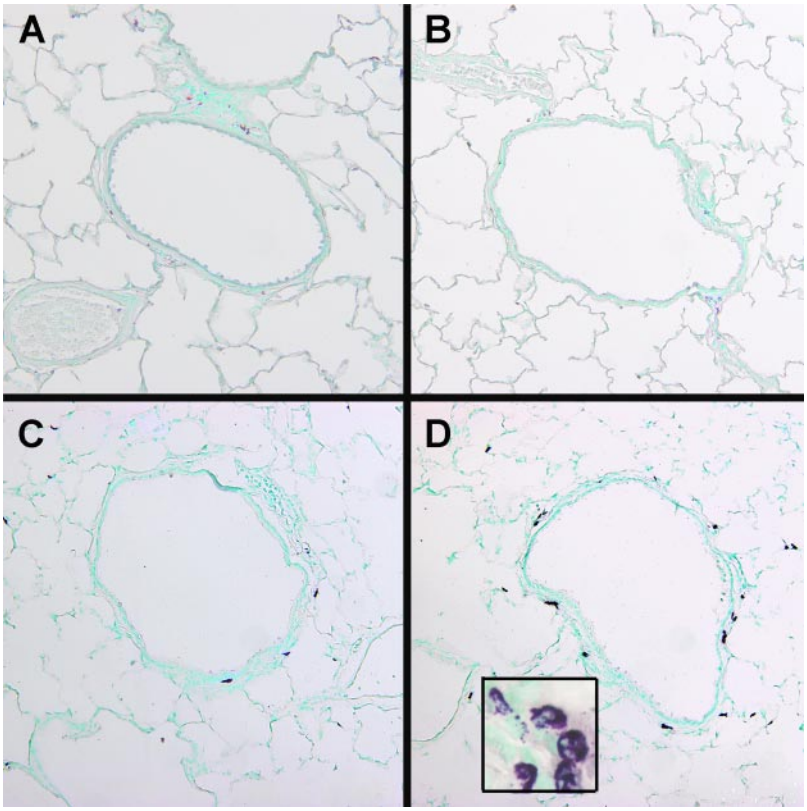


Fig. 3. Eosinophil MBP staining in lung sections. *A*: C-PBS. *B*: C-Alt. *C*: V-PBS. *D*: V-Alt. *Inset*: higher magnification of an MBP-stained area of airway wall, showing intracellular location. Airways shown have basement membrane perimeters of 1,048–1,208 μm.

and analyzed with treatment group and airway size category as independent variables, along with rat nested by group to account for multiple airways measured from each rat. From this analysis a single MBP or collagen index was obtained for each rat as a least squares mean, and planned post hoc treatment group comparisons were conducted with Fisher's least significant difference test. The number of alveolar attachments was found to vary linearly with basement membrane perimeter, and so group differences in alveolar attachments were tested with rat nested by group as a categorical variable along with treatment group and basement membrane perimeter as a covariant. A residuals analysis was conducted for each ANOVA to confirm that the residuals were normally distributed (linear normal probability plot), randomly distributed throughout the range of estimates, and randomly distributed among each of the categorical independent variables.

RESULTS

Eosinophilic inflammation. Repeated low-level Alt exposure did not result in significant persistent increases in air space inflammatory cells, as measured by the numbers of neutrophils, eosinophils, macrophages, and lymphocytes obtained by right lung BAL 5 days after the final exposure. Of interest, however, was the absence of eosinophils in the lavage samples from the Alt-exposed postbronchiolitis rats (Fig. 2*A*; $P = 0.002$ vs. Alt-challenged control inoculation rats and $P = 0.059$ vs. PBS-challenged postbronchiolitis rats, Mann-Whitney).

In contrast to the absence of eosinophils in the air space, the airway walls of the Alt-challenged postbronchiolitis rats averaged more than a sixfold increase in area staining for eosinophil MBP, compared with the PBS-challenged postbronchiolitis rats and with the Alt-challenged control inoculation rats (Figs. 2*B* and 3 $P < 0.0001$; ANOVA). Most of the airway wall MBP stain was in cells with nuclei having the characteristic morphology of eosinophils (Fig. 3*D*, *inset*), although some MBP staining was separate from any visible cell nuclei. However, the Alt-challenged control inoculation rats did not exhibit increased MBP staining in the airway wall, instead having slightly less airway wall MBP staining than the PBS-challenged rats of the same inoculation group at 5 days after the last Alt exposure (Fig. 2*B*; $P < 0.0001$, ANOVA).

Airway remodeling. Airway fibrosis was assessed as the amount of collagen staining in the airway walls, stratified by airway size. Figure 4*A* summarizes the airway wall collagen index computed for each rat. The postbronchiolitis rats had significantly more collagen-staining area in airway walls compared with the control inoculation rats ($P < 0.0001$, ANOVA); however, Alt exposure did not cause a significant additional increment of collagen staining in the airways of either inoculation group.

Exposure of the postbronchiolitis rats to Alt increased the numbers of goblet cells in small airways of 500- to 1,500-μm perimeter (Figs. 4*B* and 5 $P = 0.046$ vs. PBS-challenged postbronchiolitis rats and $P = 0.007$ vs. Alt-challenged control inoculation rats, Mann-Whitney), but the control inoculation group rats had few small airways with goblet cells, whether or not they were exposed to Alt (Fig. 4*B*; $P > 0.2$, Alt-challenged vs. PBS-challenged rats of the control inoculation group, Mann-Whitney). Goblet cells were present more frequently in larger airways, and there was not a significant difference among treatment groups for numbers of goblet cells in airways with 1,500- to 3,000-μm perimeter ($P > 0.3$, Kruskal-Wallis).

No differences were detected in the number of alveolar attachments or the percentage of abnormal alveolar attach-

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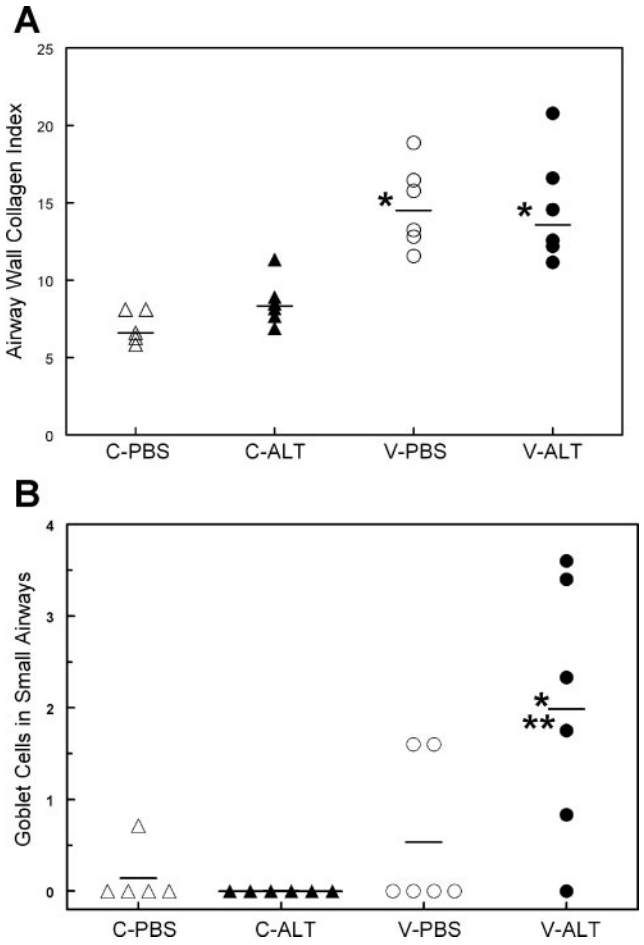


Fig. 4. Airway remodeling. *A*: airway wall collagen index for individual rats. *B*: average nos. of goblet cells in small airways of individual rats of each treatment group. Bars indicate group medians. *$P < 0.01$ vs. control inoculation group receiving the same challenge; **$P < 0.05$ vs. V-PBS.

ments to airways among treatment groups (least squares treatment group means 17–19 attachments per airway; $P > 0.2$, ANOVA).

Pulmonary physiology. Figure 6 illustrates the $FEV_{0.2}$ measured in individual rats at baseline and after six weekly exposures to aerosolized PBS or Alt. The $FEV_{0.2}$ is expressed as a fraction of TLC in order to be normalized to lung size (49). Over both time points the $FEV_{0.2}$ was lower in the postbronchiolitis rats compared with the sham-inoculated controls ($P < 0.0001$, repeated-measures ANOVA), confirming the presence of postbronchiolitis airway dysfunction. In both inoculation groups, exposures to Alt aerosol were associated with reduced $FEV_{0.2}$ over time, relative to the groups exposed to PBS ($P = 0.0009$, repeated-measures ANOVA). Although the Alt-associated declines in $FEV_{0.2}$ appear to be larger in the sham-inoculated controls than in the postbronchiolitis rats, the reductions in $FEV_{0.2}$ relative to the respective PBS-challenged groups were equivalent ($P > 0.9$, inoculation group-by-challenge group interactive effect, repeated-measures ANOVA), reflecting the tendency of the PBS-challenged postbronchiolitis rats to increase $FEV_{0.2}$ during the study period. The Alt-associated reductions in $FEV_{0.2}$ also were present at the study midpoint ($P = 0.005$, repeated-measures ANOVA), not changing significantly during the remaining 3 wk of Alt exposure. The FVC was reduced in postbronchiolitis rats at baseline (postbronchiolitis rats FVC 78 ± 2.6% TLC vs. control inoculation rats 88 ± 1.7% TLC; mean ± SD; $P < 0.0001$, *t*-test), confirming previous studies (49); however, exposures to Alt did not have a significant effect on the FVC in either inoculation group.

In contrast to Alt exposures affecting $FEV_{0.2}$ in both postbronchiolitis and sham-inoculated control rats, only the postbronchiolitis group exposed to Alt had changes in lung elastic recoil. The quasi-static pressure-volume measurements were conducted only at the final evaluation in the study. Figure 7 illustrates the pressure at the airway opening at 60%, 70%, and 80% TLC during slow deflation from TLC, representing respiratory system elastic recoil at these volumes. Although recoil was not affected by Alt exposure in the control inoculation group ($P > 0.6$, repeated-measures ANOVA), it was significantly reduced in the Alt-challenged postbronchiolitis rats ($P = 0.016$ vs. PBS-challenged postbronchiolitis rats and $P = 0.006$ vs. Alt-challenged control rats, repeated-measures ANOVA for pressures measured at all 3 volumes in each rat).

DISCUSSION

The results of this study indicate that, at low levels of *Alternaria* allergen exposure that cause minor airflow limitation in rats starting with normal airways, the rats having preexisting postbronchiolitis airway pathology respond to the Alt with persistent airway wall eosinophilic inflammation, goblet cell hyperplasia, and loss of lung elastic recoil. These observations are consistent with the hypothesis that asthmatic airways may be more vulnerable to the effects of repeated allergen exposure.

Evidence for altered eosinophil trafficking is found in the relative numbers of eosinophils in the BAL samples and the airway walls of the postbronchiolitis rats exposed to Alt. Although there was no increase in either air space or airway wall eosinophil numbers at 5 days after the last Alt challenge in the rats that did not have viral bronchiolitis early in life, the postbronchiolitis rats had elevated airway wall eosinophils but no eosinophils appearing in the BAL sample, suggesting that normal patterns of eosinophil egression had been altered. Although the regulation of egression of eosinophils from the airway wall is not well defined, some of the cells are known to enter the lymphatic system (11, 45), and this route could serve as an alternative to migrating into the air space from the airway wall. Other means of eosinophils disappearing from airway walls could include apoptosis or cytolysis (12). However, apoptotic eosinophils occur infrequently in the airway walls of rats (51), and the absence of extensive extracellular MBP in the airway wall in either of the groups of Alt-challenged rats also does not support cytolysis as a prominent means of eosinophil clearance, in contrast to the diffuse extracellular MBP that has been described in affected areas of atopic dermatitis in humans (26). Thus we propose that the persistent airway wall eosinophilia in the postbronchiolitis Alt-exposed rats represents a population of eosinophils that are not egressing from the airway wall normally.

A similar pattern of increased airway wall eosinophils and reduced air space eosinophils after repeated allergen exposure has been observed in mice treated with a metalloproteinase inhibitor and in mice having gene deletions for matrix metalloproteinase-2 or combined deletions for matrix metalloproteinases-2 and -9 (8,

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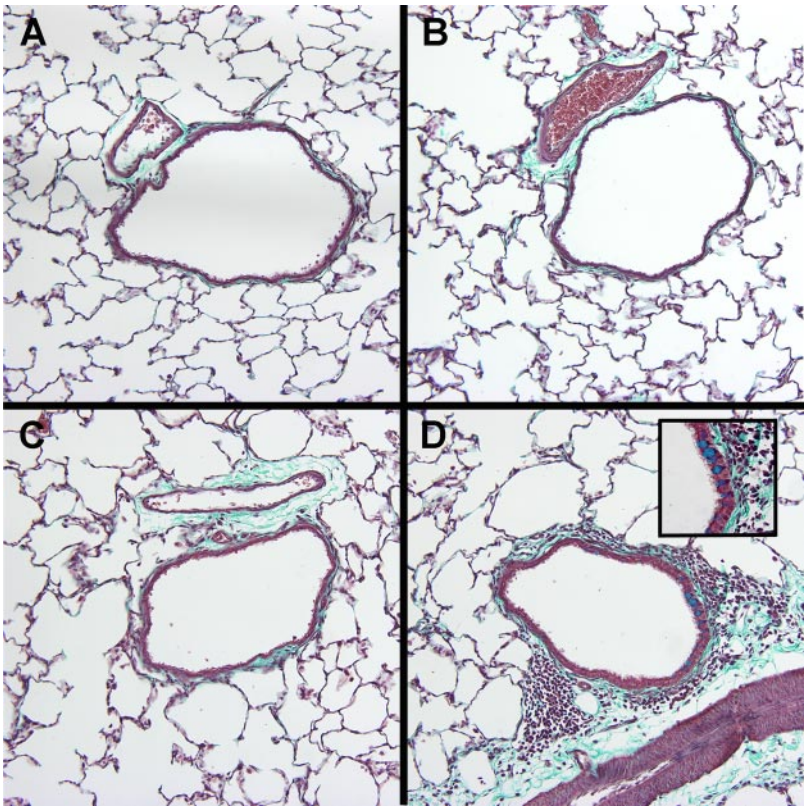


Fig. 5. Modified trichrome stains of lung sections, showing collagen (green) and goblet cells (blue). *A*: C-PBS. *B*: C-Alt. *C*: V-PBS. *D*: V-Alt. *Inset*: higher magnification, showing goblet cells and airway wall collagen staining. Airways shown have basement membrane perimeters of 979–1,138 μm.

9). Reduced BAL fluid concentrations of chemokines, along with normal migration of the lung eosinophils to chemokine gradients in vitro, suggest that the altered eosinophil egression in these metalloproteinase-knockout models is due to loss of metalloproteinase-dependent transepithelial gradients of eotaxin and other CCL chemokines (8, 9). In humans, a comparison of local inflammatory responses 48 h after segmental allergen challenge found that subjects with asthma had significantly less metalloproteinase-9 in the challenged segment compared with nonasthmatic allergic subjects, although at 48 h after a single allergen challenge the numbers of air space eosinophils did not differ between the groups (22). It is plausible that the magnitude and/or timing of chemokine secretion into the air space may be altered in asthmatic airways during chronic exposure to allergen, resulting in persistence of airway wall eosinophils.

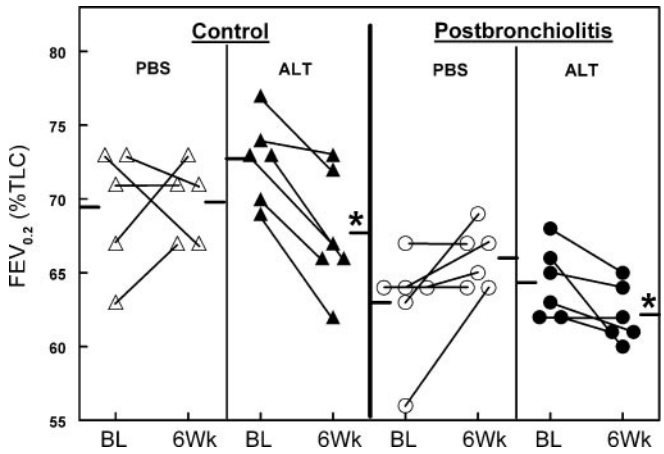


Fig. 6. Changes in airflow limitation. Forced expiratory volume in 0.2 s [$FEV_{0.2}$; normalized to total lung capacity (TLC)] in individual rats at baseline (BL) and after 6 weekly exposures to aerosolized PBS or Alt. Small bars indicate group means at each time point. *$P < 0.001$ vs. respective PBS group changes.

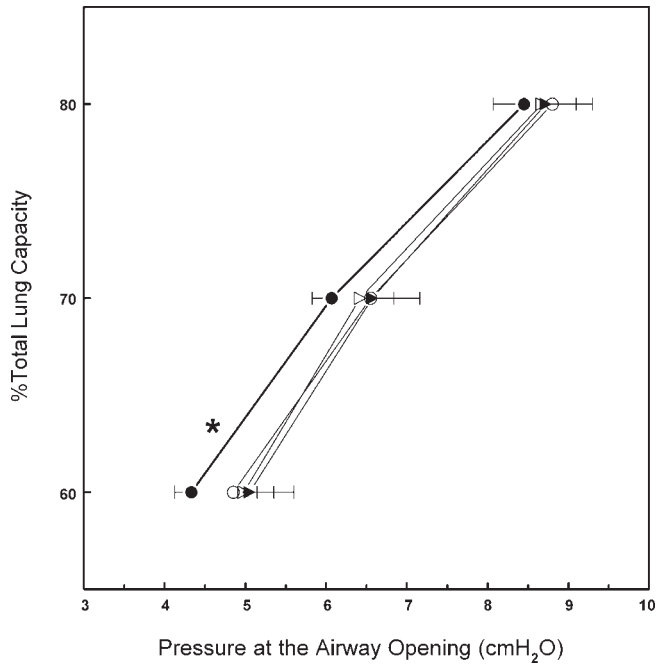


Fig. 7. Elastic recoil. Pressures at the airway opening, measured at 3 lung volumes during slow deflation from TLC, after 6 wk of aerosol challenges. Each symbol is group mean ± SD (group symbols as in Fig. 6). *$P \leq 0.016$, V-Alt vs. V-PBS or C-Alt, comparing pressures at all 3 volumes.

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Physiological changes associated with Alt exposure included a significant, but small, reduction in $FEV_{0.2}$, which occurred to a similar extent in both postbronchiolitis and control-inoculation groups, and reduced lung elastic recoil, which occurred only in the postbronchiolitis rats. Previously, we reported (49) that the chronic airway obstruction in rats with postbronchiolitis airway dysfunction has components of both airflow limitation and premature airway closure, the airway closure component (detected as reduced FVC) being the prominent factor contributing to reduced $FEV_{0.2}$ in the postbronchiolitis rats. In the present study, the postbronchiolitis rats had the expected reduced FVC compared with the control-inoculation group, but there were no additional changes in FVC associated with Alt exposure; that is, the Alt-induced changes in $FEV_{0.2}$ appeared to be due to a new airflow limitation component and not due to an exacerbation of the preexisting postbronchiolitis airway closure pathology. The significance of this observation is that Alt-induced airflow limitation likely developed via a different underlying pathophysiology (location and/or mechanism) than the chronic obstructive process that develops after recovery from bronchiolitis in these rats. The reduced lung elastic recoil in the postbronchiolitis/Alt-challenged rats likely contributed to the airflow limitation in this group, but the control/Alt-challenged group also developed some airflow obstruction without a change in recoil or in FVC, consistent with reduced caliber of airways proximal to the sites of airway closure at lower lung volumes.

Lung elastic recoil was reduced only in the rats that had both the postbronchiolitis airway dysfunction and exposure to Alt. This is of interest, not only as an indicator of increased vulnerability of asthmalike airways to subsequent allergen exposure, but also as a possible mechanism having relevance to severe asthma. The elastic forces of lung parenchyma often are reduced in persons with asthma, particularly during exacerbations, but also during stable periods of moderate-severe persistent asthma (7, 16, 54). Physiologically this is important, in that lung elastic recoil promotes airflow both by creating a pressure gradient on alveolar air (30, 37) and by transmitting a bronchodilating force to the airways (7). Reduced lung recoil in asthmatics has been identified as a component of airflow limitation (14, 29) and as a predictor of near-fatal episodes (13) and is attracting renewed interest as a distinguishing feature of severe asthma (53). One proposed mechanism for reduced elastic recoil is disruption of elastin in lung parenchyma (3, 28), and elevated levels of neutrophil elastase have been detected in asthmatics who have increased neutrophils in their sputum (52). Alveolar macrophages also produce elastases (41), and in a rat model of tobacco smoke-induced emphysema the pathogenetic elastolysis is dependent on macrophages (33, 34). Desmin is another cytoskeletal protein that contributes to lung elastic recoil (42), although it is not known whether this protein is altered by inflammatory processes. Airways from persons with fatal asthma exhibit alterations of alveolar attachments and elastin fibers in airways and peribronchial alveoli, which could contribute to airway-parenchymal uncoupling and obstruction (27, 28); however, we did not find histological evidence of disruption of alveolar attachments in the rats exhibiting reduced elastic recoil after allergen exposure in the present study. Although the changes in lung recoil were not severe within the 6-wk course of low-level allergen exposures used in the present study, our model of reduced lung elastic recoil in postbronchiolitis rats exposed to Alt is unique in its analogy to asthmatic airways interacting with allergen exposures, and it therefore may have value for the study of mechanisms potentially relevant to severe persistent asthma or asthma exacerbations in humans.

We selected Alt as the allergen challenge for this study to mimic exposure to an allergen that has been identified as a risk factor for the inception of asthma (10, 18) as well as a risk factor for severe exacerbations of asthma (32). *Alternaria* species produce proteases that may enhance allergic sensitization, as well as having direct proinflammatory effects on epithelial cells and eosinophils via protease-activated receptors and G protein-mediated exocytosis (20, 23, 38). These innate inflammatory mechanisms may interact with concomitant IgE- and Th2-mediated allergic responses to fungal proteins to create exaggerated asthma-associated airway pathology (38).

In conclusion, the results of this study demonstrate that preexisting postbronchiolitis pathology alters the response to low-level allergen exposure and results in qualitatively different allergen-induced pulmonary pathophysiology compared with control rats that have normal airways before allergen challenges.

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GRANTS

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