The Importance of Leukotrienes in Airway Inflammation in a Mouse Model of Asthma

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Summary

Inhalation of antigen in immunized mice induces an infiltration of eosinophils into the airways and increased bronchial hyperreactivity as are observed in human asthma. We employed a model of late-phase allergic pulmonary inflammation in mice to address the role of leukotrienes (LT) in mediating airway eosinophilia and hyperreactivity to methacholine. Allergen intranasal challenge in OVA-sensitized mice induced LTB_4 and LTC_4 release into the airspace, widespread mucus occlusion of the airways, leukocytic infiltration of the airway tissue and bronchoalveolar lavage fluid that was predominantly eosinophils, and bronchial hyperreactivity to methacholine. Specific inhibitors of 5-lipoxygenase and 5-lipoxygenase-activating protein (FLAP) blocked airway mucus release and infiltration by eosinophils indicating a key role for leukotrienes in these features of allergic pulmonary inflammation. The role of leukotrienes or eosinophils in mediating airway hyperresponsiveness to aeroallergen could not be established, however, in this murine model.

IgE-receptor-mediated activation of mast cells in the airways leads to oxygenation of arachidonic acid by 5-lipoxygenase and generation of leukotrienes (LT)[1] (1, 2). LTB_4 stimulates leukocyte chemotaxis (3), and the cysteinyl leukotrienes C_4, D_4, and E_4 induce bronchoconstriction (4, 5), mucus secretion (2) and also promote eosinophil chemotaxis (6, 7) and hydrostatic pulmonary edema formation (8, 9). After inhalation of specific allergen, allergic individuals may have both an early and late asthmatic response. Mast cell-derived bronchoconstrictors such as histamine (10) and cysteinyl leukotrienes (11, 12) likely contribute to the early bronchospastic response occurring within 30 min after allergen challenge. A late asthmatic phase occurs several hours after exposure to allergen and is of prolonged duration; an infiltration of the airway epithelium and bronchial fluid by eosinophils is characteristic of this phase (13, 14).

The mouse is also susceptible to development of IgE-mediated allergic responses. The late-phase influx of eosinophils is reproduced in a murine model in which allergic airway disease develops after ovalbumin inhalation in mice previously sensitized to ovalbumin intraperitoneally (15). Increased airway responsiveness to methacholine (16) or acetylcholine (17) challenge also occurs in immunized mice after airway exposure to antigen. In a mouse model of allergen-induced airway inflammation, we examined the role of leukotrienes in mediating airway eosinophil infiltration, mucus release, and hyperresponsiveness to methacholine. We report that leukotrienes are key mediators of the mucus release and eosinophil infiltration of the airways, but not the airway hyperresponsiveness of the late phase asthma response.

Materials and Methods

Special Reagents. Crystalline OVA was obtained from Pierce Chem. Co. (Rockford, IL), aluminum potassium sulfate (alum) from Sigma Chem. Co. (St. Louis, MO), pyrogen-free distilled water from Baxter, Healthcare Corporation (Deerfield, IL), 0.9% sodium chloride (normal saline) from Lyphomed (Deerfield, IL), and Trappsol™ HPB-L100 (aqueous hydroxpropyl beta cyclodextrin; 45 wt/vol% aqueous solution) from Cyclodextrin Technologies Development Inc. (Gainesville, FL). The OVA (500 μg/ml in normal saline) was mixed with equal volumes of 10% (wt/vol) alum in distilled water. The mixture (pH 6.5 using 10 N NaOH) after incubation for 60 min at room temperature underwent centrifugation at 750 *g* for 5 min; the pellet was resuspended to the original volume in distilled water and used within 1 h.

[1] *Abbreviations used in the paper:* alum, aluminum potassium sulfate; BAL, bronchoalveolar lavage; FLAP, 5-lipoxygenase-activating protein; i.n., intranasal; LT, leukotriene; PAS, periodic acid–Schiff.

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The selective 5-lipoxygenase inhibitor zileuton (*N*-[1-benzo [*b*]thien-2-ylethyl]-*N*-hydroxyurea) (18) was kindly provided by Drs. Randy L. Bell and George W. Carter (Abbott Laboratories, Abbott Park, IL). The selective 5-lipoxygenase-activating protein (FLAP) inhibitor MK-886 (L-663,536;3-[1-(4-chlorbenzyl)3-t-butyl-thio-t-isopropyl-indol-2-yl]-2-2-dimethylpropanoic acid) (19, 20) was generously provided by Dr. Anthony Ford-Hutchinson (Merck Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Québec, Canada). Zileuton was dissolved in Trappsol™; MK-886 was dissolved in dimethylsulfoxide (DMSO) and then diluted in normal saline, pH 7.0.

Animals. All animal use protocols were approved by the University of Washington Animal Care Committee. Female BALB/c mice (6–8 wk of age at purchase; D and K, Seattle, WA) were housed under conventional conditions for the studies.

Allergen Immunization/Challenge Protocol. Mice received an i.p. injection of 0.2 ml (100 μg) of OVA complexed with alum on day 0 and 14. On days 14, 25, 26, and 27, mice were anesthetized with 0.2 ml i.p. of ketamine (0.44 mg/ml)/xylazine (6.3 mg/ml) in normal saline before receiving an intranasal (i.n.) dose of 100 μg OVA in 0.05 ml normal saline on day 14 and an i.n. dose of 50 μg OVA in 0.05 ml normal saline on days 25, 26, and 27. Two control groups were used. The first group received normal saline with alum i.p. on days 0 and 14 and normal saline without alum i.n. on days 14, 25, 26, and 27. The second group received OVA with alum i.p. on days 0 and 14, OVA without alum i.n. on day 14, and normal saline alone on days 25, 26, and 27.

Leukotriene Inhibitor and LTD_4 Studies. To assess the role of 5-lipoxygenase products in airway inflammation, the 5-lipoxygenase inhibitor zileuton (35 mg/kg) or the FLAP inhibitor MK-886 (3 mg/kg) were given i.p. 30 min before each i.n. challenge on days 25, 26, and 27. In one set of animals, zileuton was also given on days 0 and 14 (before i.p. OVA). Zileuton at 35 mg/kg inhibits cysteinyl leukotriene release by ~95% in passively sensitized rats given BSA antigen i.p. (18). MK-886 at 3 mg/kg inhibits LTB_4 biosynthesis by 98% in inflamed rat paws given subplantar injection of thioglycollate followed 6 h later by calcium ionophore A23187 treatment (20).

To assess the role of leukotrienes in airway mucus release, non-immunized mice received 0.1 ml i.n. of either LTD_4 (2.5 μg in 1% ethanol) or vehicle (1% ethanol). After 60 min, lung tissue was obtained and stained with hematoxylin and eosin as described below.

Pulmonary Function Testing. On day 28, 24 h after the last i.n. administration of either normal saline or OVA, pulmonary mechanics to intravenous infusions of methacholine were determined in mice in vivo by a plethysmographic method modified from that previously described by Amdur and Mead (21) and Martin et al. (22). After pentobarbital (70–90 mg/kg, i.p.) anesthesia, the mice underwent thoracotomy with wide opening of the thorax, jugular vein cannulation with 8 cm silastic tubing, and tracheal intubation with an angled 18-gauge blunt needle. In one compartment (0.25 ml dead space) of a two chamber whole body plethysmograph, the animal was placed in a supine position and connected to a Harvard ventilator. Normal arterial blood gases were maintained by the following minute ventilation: tidal volume = 0.2 ml/20 gm, frequency 120 breaths/min, positive end-expiratory pressure 2.5–3 cm H_2O (23). Constant temperature in the plethysmograph was maintained by a copper mesh heat sink. Since the plethysmograph was a closed system, the change in box pressure (P_{box}) of the first chamber (measured by a sensitive transducer [±0.7 cm H_2O]) represented the change in lung volume ($\Delta vol = P_{box}$) of the mouse; P_{box} is equivalent to lung pleural pressure. The system was calibrated by syringe delivery of 0.2 ml air at a frequency of ~120/min. Rapid ambient pressure swings (e.g., door openings) in the first chamber were offset by reference of the transducer to the pressure in the second chamber. A microvalve with a time constant ~4.5 s was placed between the two compartments to correct for any temperature increases in the first chamber resulting from the body heat of the animal. A second microvalve with a longer time constant (~11 s) was open to ambient air to keep mean P_{box} close to zero. Pressure movements at the opening of the tracheal tube (P_{aw}) were measured by another transducer, referenced to P_{box} to determine transpulmonary pressure ($P_{tp} = P_{aw} - P_{box}$).

After initial inflation to a P_{aw} of 30–35 cm H_2O, the lungs were inflated at least once 1–2 min before each measurement to prevent partial collapse. An analog-to-digital data acquisition system (Strawberry Tree®) was employed to sample P_{tp} and P_{box} at 5-ms intervals. The digital P_{tp} data were then offset by 15 ms to bring it into phase with the P_{box} signal as determined by making a step change in P_{box} with no animal connected to the tracheal cannula. A smoothing function was applied to dampen the background noise in P_{box}

$$\left[\frac{(X-1)+2X+(X+1)}{4}\right],$$

where X is the flow at a specific time point). The change in volume from point-to-point/5 ms was used to calculate flow.

Data from seven consecutive breaths collected three times during the first 10 min were used to calculate basal pulmonary function. Methacholine was then infused by hand delivery into the jugular vein over 10 s at increasing concentrations (3, 12, 48, 120, and 480 mg/kg) after 10 min of ventilation. Intervals of 1.5–3 min between injections enabled pulmonary function to return to basal levels. Resistance (R), lung conductance ($G_L = 1/R$), and dynamic compliance (C_{dyn}) were determined for both the control period and the peak response to each methacholine concentration that was seen during 30 s of monitoring post-injection to bracket the peak response. Tracheal tube resistance (0.63 cm $H_2O \times ml^{-1} \times s$) was subtracted from all airway resistance measurements. The difference in P_{tp} and air flow at mid-tidal volume on inflation and deflation was used to calculate R. C_{dyn} was calculated as the change in tidal volume divided by the difference between P_{tp} at end-inspiration and end-expiration when flow is zero ($C_{dyn} = \Delta Vt/\Delta P_{tp}$). At each methacholine concentration, G_L and C_{dyn} were expressed as the percent of basal value. The methacholine dose which reduced G_L and C_{dyn} to 75 and 50% of the control values (ED_{25} and ED_{50}, respectively) was calculated from the log dose-peak response curves and expressed as the geometric mean + log SD At the completion of pulmonary function testing, each mouse was exsanguinated by cardiac puncture.

Bronchoalveolar Lavage. After tying off the left lung at the mainstem bronchus, the right lung was lavaged three times with 0.4 ml of normal saline. Bronchoalveolar lavage (BAL) fluid cells from a 0.05-ml aliquot of the pooled sample were counted using a hemocytometer and the remaining fluid centrifuged at 4°C for 10 min at 200 *g*. The supernatant was stored at −70°C until eicosanoid analyses were performed. After resuspension of the cell pellet in normal saline containing 10% BSA, BAL cell smears were made on glass slides. To stain eosinophils, dried slides were stained with Discombe's diluting fluid (0.05 % aqueous eosin and 5% [vol/vol] acetone in distilled water) (24) for 5–8 min, rinsed

with water for 0.5 min, and counterstained with 0.07% methylene blue for 2 min.

Lung Histology. After BAL, the trachea and left lung (upper and lower lobes) were obtained and fixed in Carnoy's solution at 20°C for ~15 h. After embedding in paraffin, the tissues were cut into 5-μm sections and stained with Discombe's solution and counter-stained with methylene blue as described above. The eosinophil number per unit airway area (2,200 μm^2) was determined by morphometry as previously described (25, 26). Airway mucus was identified by the following staining methods: methylene blue, hematoxylin and eosin, mucicarmine, toluidine blue, alcian blue, and alcian blue/periodic acid-Schiff (PAS) reaction (27, 28). Mucin was stained with mucicarmine solution; metanil yellow counterstain was employed. Mucin and sialic acid-rich nonsulfated mucosubstances were stained metachromatically with toluidine blue, pH 4.5. Acidic mucin and sulfated mucosubstances were stained with alcian blue, pH 2.5; nuclear fast red counterstain was used. Neutral and acidic mucosubstances were identified by alcian blue, pH 2.5, and PAS reaction. The degree of mucus plugging of the airways (0.5–0.8 mm in diameter) was also assessed by morphometry. The percent occlusion of airway diameter by mucus was classified on a semiquantitative scale from 0 to +++++ as described in the Figure Legends. The histologic and morphometric analyses were performed by individuals blinded to the protocol design.

Assay of Airway Mucus Glycoproteins. Mucus glycoproteins in BAL fluid were assayed by slot blotting and PAS staining as previously described (29, 30). Nitrocellulose membranes (0.2-μm pore size; Schleicher & Schuell, Keene, NH) were wetted in distilled water and then in normal saline before placement in a Minifold II 72-well slot blot apparatus (Schleicher & Schuell). The BAL fluid samples (0.05 ml) and aliquots (0.05–0.75 ml) of a stock solution (2 μg/ml) of human respiratory mucin glycoprotein (31) (kindly provided by Dr. James Shelhamer) were blotted onto the nitrocellulose membranes by water suction vacuum, and mucus glycoproteins were visualized by PAS reaction. Reflectance densitometry was performed to quantitate the PAS staining. The images were captured and digitized by a ScanJet IIcx Scanner with HP DeskScan II software (Microsoft® Windows™ Version) (Hewlett Packard, Palo Alto, CA). This system was linked to a Dell Dimension XPS P90 computer (Dell Corporation, Austin, TX) employing Image-Pro® Plus, Version 1.1 for Windows™ software (Media Cybernetics, Silver Spring, MD). The images were assessed on a 256 gray level scale using a Dell UltraScan 17ES monitor with extra high-resolution graphics mode (1,280 × 1,024 pixels, 78.9-kHz horizontal scanning frequency, 74-Hz vertical scanning frequency). The integrated intensity of the PAS reactivity of the BAL samples was quantitated by comparison to the standard curve for human respiratory mucin as previously described (29).

Specific Antibody Levels. Previous studies have demonstrated that i.p. immunization with OVA results in detectable levels of OVA-specific IgE in the blood of BALB/c mice (32). Indirect ELISA was employed to determine OVA-specific IgE serum antibody titers. ELISA plates (ICN, Costa Mesa, CA) were coated with OVA (20 mg/ml) diluted in 0.1 M $NaHCO_3$ buffer, pH 8.3 and incubated at 4°C for 18 h. After washing three times, the plates were incubated with 1% BSA in PBS, pH 7.4, at 37°C for 2 h. Serial dilutions of the serum samples in 1% BSA/PBS buffer were added to the plates and incubated at 4°C for 18 h before washing again. The wells were incubated with HRP-conjugated rat anti–mouse IgE monoclonal antibody (PharMingen, San Diego, CA)

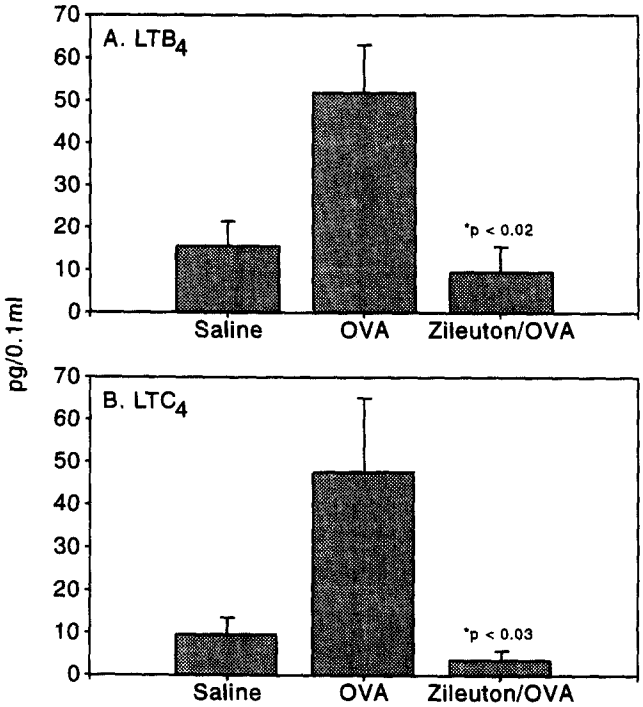


Figure 1. 5-Lipoxygenase inhibition blocks leukotriene release in OVA-immunized/challenged mice. BAL fluid was obtained on day 28 from sham-sensitized mice receiving i.n. saline challenge (*Saline*; $n = 8$), and OVA-sensitized mice either untreated (*OVA*; $n = 7$) or treated (*Zileuton/OVA*; $n = 7$) with the 5-lipoxygenase inhibitor zileuton 30 min before i.n. OVA challenge on day 25, 26, and 27. The BAL fluid was assayed for (*A*) LTB4 and (*B*) LTC4 as described in Materials and Methods. **P* value, Zileuton/OVA versus OVA.

diluted in 50% goat serum (GIBCO BRL, Gaithersburg, MD)/PBS buffer for 2 h at room temperature. 3,3′,5,5′-tetramethylbenzidine substrate was used to develop the wells with absorbance determined at 610 nm. The internal standard in each assay consisted of pooled serum from OVA-immunized BALB/c mice.

Eicosanoid Assays. BAL fluid was assayed for LTB_4 and LTC_4 in duplicate by RIA (33). LTB_4 was assayed using a commercial [^{3}H]LTB_4 RIA kit (NEN Research Products, Boston, MA). The detection limit for LTB_4 was 12.5 pg/0.1 ml sample. LTC_4 was obtained from Cayman Chemical Co. (Ann Arbor, MI) and [^{3}H]LTC_4 from NEN Research Products (Boston, MA). Rabbit antisera against LTC_4 (kindly provided by Drs. Robert W. Egan and John L. Humes, Merck Institute for Therapeutic Research, Rahway, NJ) had the following cross-reactivities at B/B_0 50%: (5*S*, 6*R*)-LTC_4, 100%; LTD_4, 43%; 11-*trans*-LTD_4, 47%; LTE_4, 6%; LTF_4, 50%; (5*R*, 6*R*)-LTC_4, 100%; (5*S*, 6*S*)-LTC_4, 1.7%; (5*R*, 5*S*)-LTC_4, 0.7%; LTC_4-sulfone, 10%; LTD_4-sulfone, 7%; LTE_4-sulfone, 0.4%; LTF_4-sulfone, 1.0%; and LTB_4, (5*S*, 6*R*)-7,8,9,10,11,12,14,15-octahydro-LTC_4, PGE_2, $PGF_{2\alpha}$, glutathione, 5-HETE, and arachidonic acid each <0.2% (34). The LTC_4 RIA had a sensitivity of 20 pg/0.1 ml sample.

Statistical Analyses. The pulmonary function data were evaluated by analysis of variance (ANOVA) using the protected least significant difference method (Statview II, Abacus Concepts, Berkeley, CA). This method uses a multiple *t* statistic to evaluate all possible pairwise comparisons and is applicable for both equal and

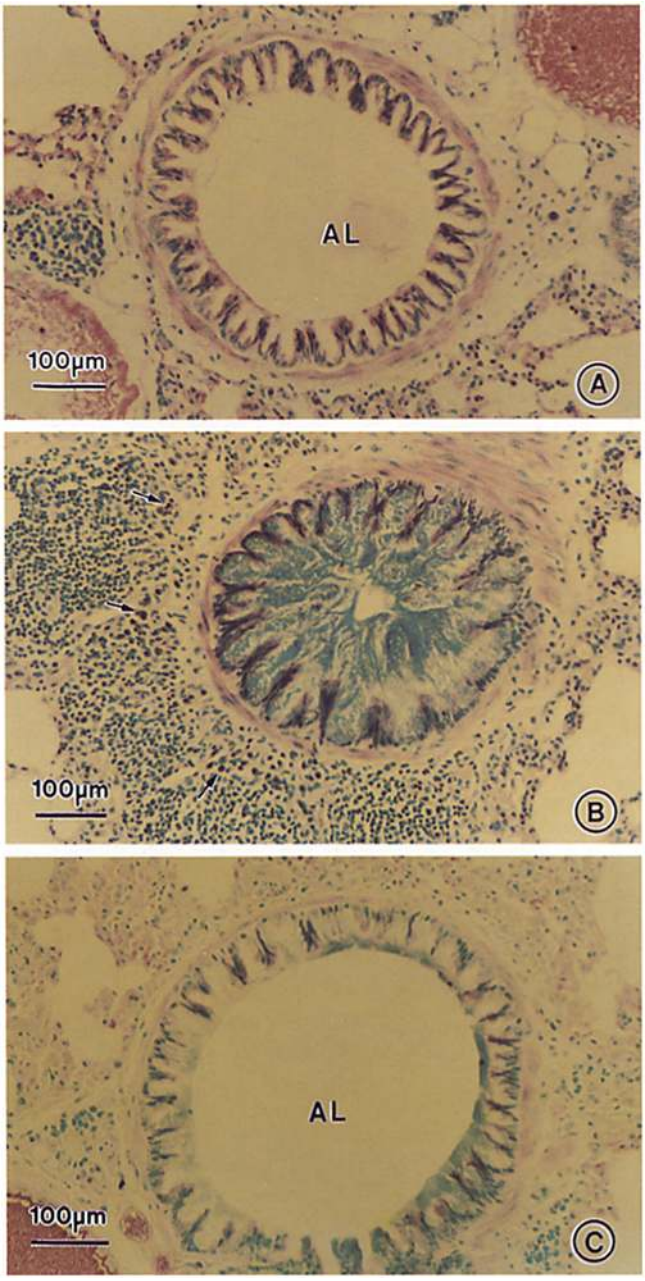


Figure 2. Histologic evidence of airway inflammation in OVA-treated and control mice: effect of 5-lipoxygenase inhibition. Lung tissue was obtained from sham-sensitized and saline-challenged mice (*A*), and OVA-immunized/challenged mice in the absence (*B*) or presence (*C*) of zileuton treatment. Lung sections were stained in Discombe's solution, counterstained with methylene blue, and examined by light microscopy as described in Materials and Methods. (*A*) The airway lumen (*AL*) is clear of mucus and cells in control mice. The bronchial epithelium is intact. Connective tissue cells but not leukocytes are present in the peribronchial interstitial space. (*B*) Large amounts of secreted mucus are identified in the lumen of the airways of the OVA-sensitized/challenged mice by methylene blue staining. Massive infiltration of the interstitial tissue by eosinophils and other inflammatory cells is noted (*arrows*). (*C*) Airway mucus release in the airway lumen (*AL*) is markedly reduced when the 5-lipoxygenase inhibitor zileuton is given before i.n. OVA. The infiltration of the interstitial tissue by eosinophils is also reduced after zileuton treatment compared to OVA-challenge alone (*B*). Bars, 100 μm.

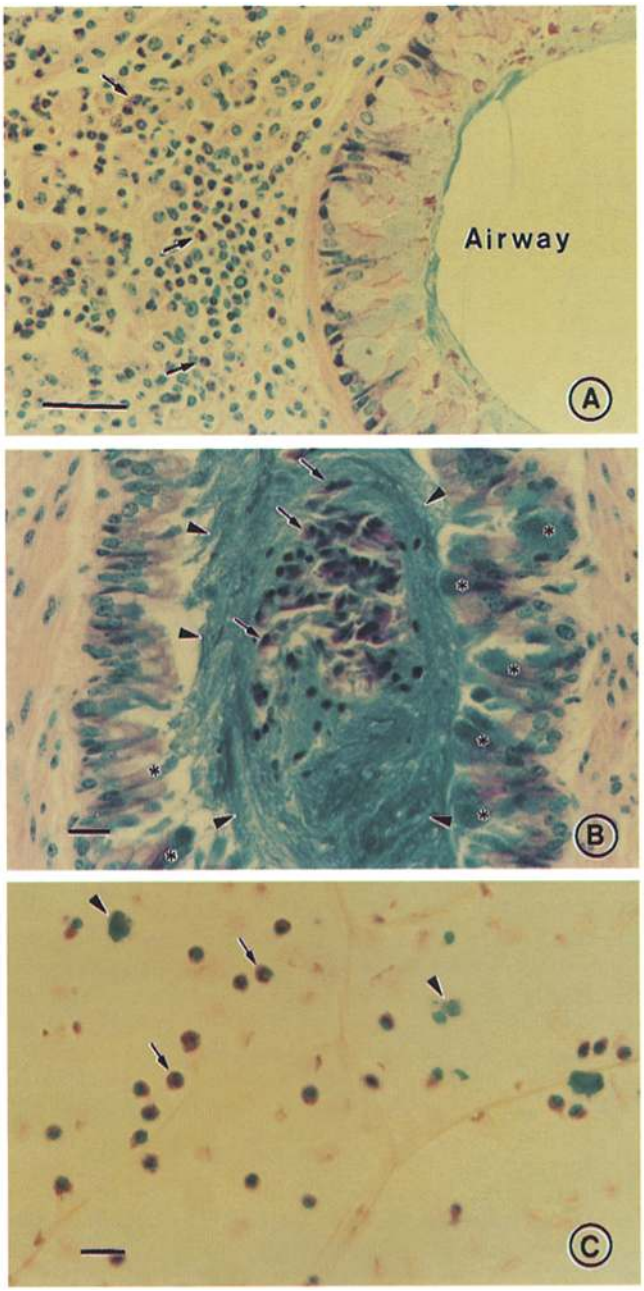
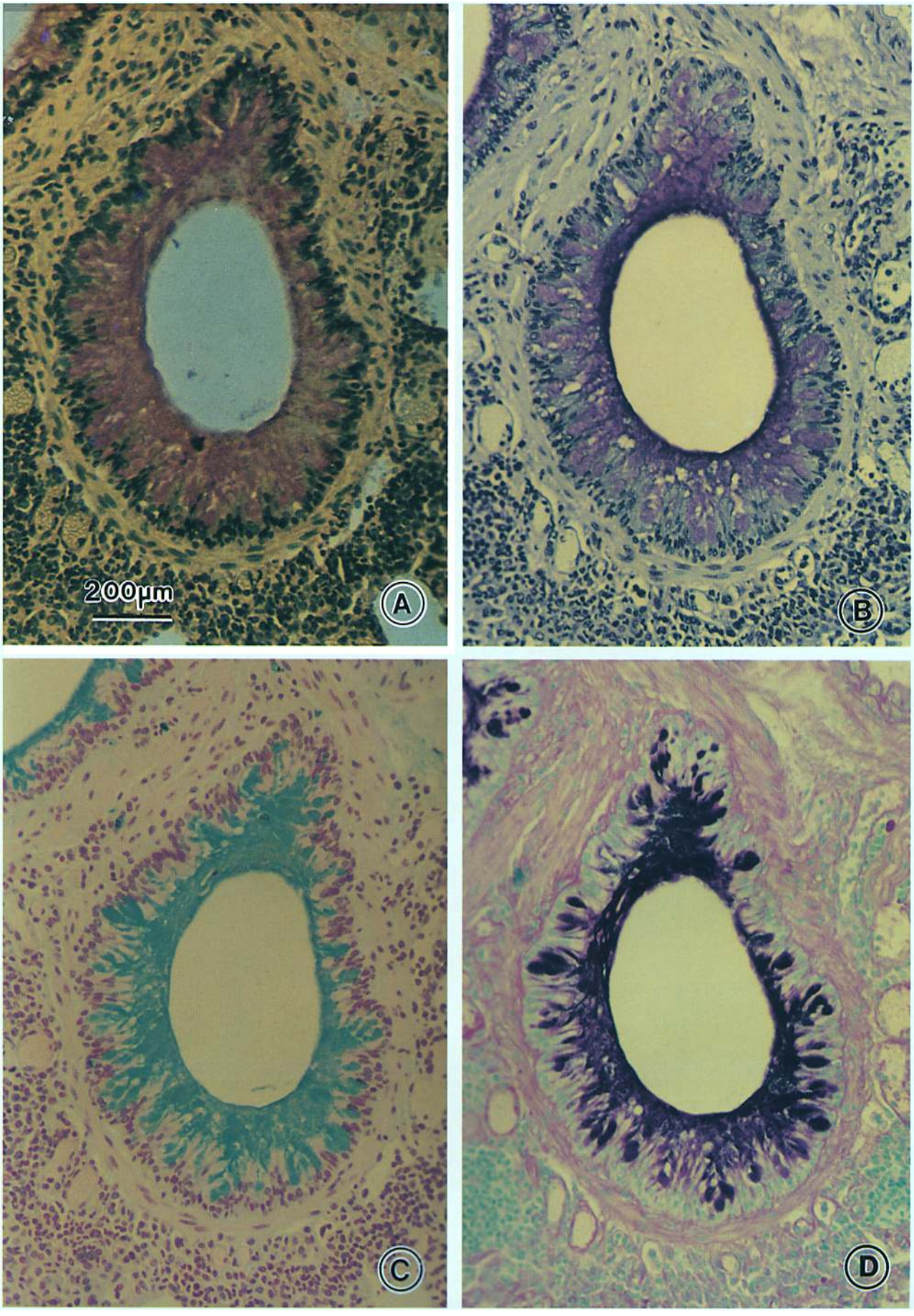


Figure 3. Eosinophil infiltration of lung tissue and BAL fluid in OVA-treated mice. Lung tissue and BAL fluid cells from OVA-immunized/challenged mice were stained for eosinophils as described in Fig. 2 *B*. (*A*) Eosinophils (*arrows*) are the predominant inflammatory cell in the interstitial tissue of the airway. (*B*) Large accumulation of mucus (*arrowheads*) in the airway lumen is observed. Many goblet cells (*) are seen in the airway. Numerous eosinophils (*arrows*) are found in the mucus plug. (*C*) The majority of the BAL fluid cells are eosinophils (*arrows*). Alveolar macrophages (*arrowheads*) are also observed. Bars, (*A*) 50 μm; (*B* and *C*) 10 μm.

Figure 4. Mucus occlusion of lower airways in OVA-treated mice. Lower lobe tissue of the left lung from OVA-immunized/challenged mice was stained for mucus by mucicarmine (*A*), toluidine blue (*B*), alcian blue (*C*), and alcian blue/PAS (*D*) procedures as described in Materials and Methods. (*A*) Airway mucin is stained rose-red by mucicarmine, and interstitium is stained yellow by the metanil yellow counterstain. (*B*) Mucin and nonsulfated acidic mucosubstances are stained metachromatically red-purple by toluidine blue. (*C*) Acidic sulfated mucosubstances and mucins are stained blue by alcian blue, and nuclei are stained pink-red by the nuclear fast red counterstain. (*D*) Acidic and neutral mucosubstances are stained magenta by alcian blue/PAS. Bar, 100 µm.


200μm
A
B
C
D

unequal pair sizes. The other data are reported as the mean ± SE of the combined experiments. Differences were analyzed for significance ($P < 0.05$) by Student's two-tailed t test for independent means.

Results

Allergen-Specific IgE Production. OVA-specific IgE (14.9 ± 3.5 U/ml, $n = 5$) was detected on day 28 in the blood of mice given i.p. OVA and alum on day 0 and 14 and i.n. OVA on days 25, 26, and 27. In contrast, control mice treated with i.p. saline and alum and i.n. saline ($n = 6$) had no detectable anti-OVA IgE.

Allergen-induced Eicosanoid Release in BAL Fluid. BAL fluid was obtained 24 h after the third i.n. administration of either OVA or saline in mice that had received i.p. either OVA or saline respectively and assayed for eicosanoids. The BAL fluid levels of the 5-lipoxygenase products LTB_4 (Fig. 1 *A*) and LTC_4 (Fig. 1 *B*) were 3.4-fold ($P < 0.03$ compared to saline) and 5.0-fold ($P < 0.05$, compared to saline) higher respectively in the OVA-immunized/challenged mice than in the mice receiving saline only. The selective 5-lipoxygenase inhibitor zileuton given 30 min before i.n. challenge with OVA on day 25, 26, and 27 significantly inhibited LTB_4 and LTC_4 release compared to mice challenged with i.n. OVA in the absence of zileuton (Fig. 1). The levels of LTB_4 and LTC_4 in the BAL fluid of OVA-immunized/challenged mice treated with zileuton were not significantly different from those of the saline control group (Fig. 1).

Allergen-induced Airway Inflammation. To assess allergen-induced airway inflammation histologically, lung tissue and BAL fluid were obtained on day 28, 24 h after the last of 3 sequential i.n. OVA challenges on days 25, 26, and 27. By light microscopy, prominent infiltration of the bronchial interstitium by eosinophils was observed (Figs. 2 *B* and 3 *A*). Eosinophil influx into the bronchial epithelial mucus layer (Fig. 3 *B*) and the BAL fluid (Fig. 3 *C*) was also noted. 63.6 ± 3.2% ($n = 10$) of the BAL fluid cells were eosinophils in OVA-sensitized/challenged mice compared to 0.8 ± 0.3% ($n = 10$) in saline-challenged control animals ($P = 0.0001$). Mucus occlusion of the airways occurred in immunized mice after bronchial challenge with OVA (Fig. 2 *B*). Airway mucus release in both lower (Fig. 4) and upper (Fig. 5) pulmonary airways was identified by several histochemical staining procedures: mucin by mucicarmine stain (Figs. 4 *A* and 5 *A*), acidic nonsulfated mucosubstances by toluidine blue (Figs. 4 *B* and 5 *B*), acidic sulfated mucosubstances by alcian blue (Figs. 4 *C* and 5 *C*), and neutral and acidic mucosubstances by alcian blue/PAS reaction (Figs. 4 *D* and 5 *D*). Airway lumen occlusion by mucus was greater in the smaller diameter airways. These inflammatory changes were absent in i.n. saline-challenged control animals that had been treated i.p. with either saline (Fig. 2 *A*) or OVA (not shown) with alum.

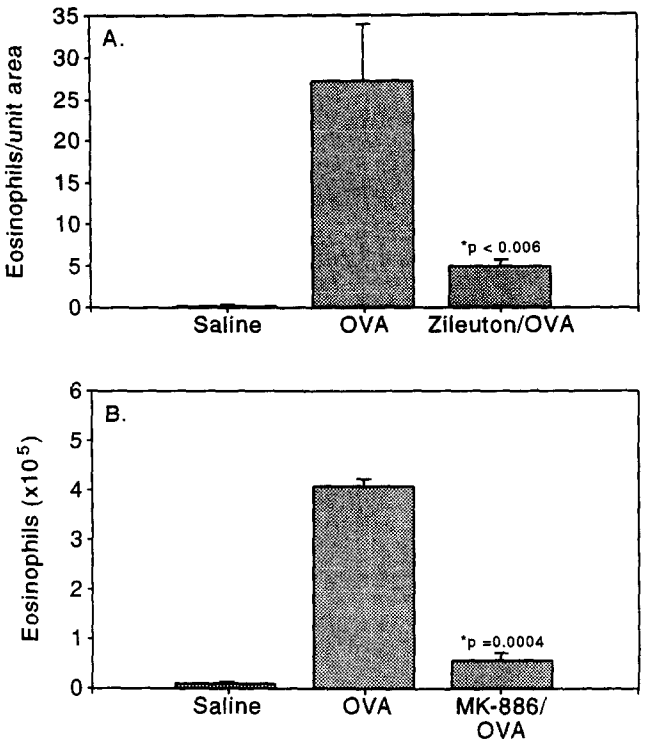


Figure 6. Leukotriene inhibition blocks eosinophil infiltration of airway tissue and BAL fluid in OVA-treated mice. (*A*) Lung tissue was obtained from sham-sensitized and saline-challenged mice (*Saline*; $n = 8$) and OVA-sensitized/challenged mice in the absence (*OVA*; $n = 7$) or presence (*Zileuton/OVA*; $n = 7$) of zileuton treatment. The number of eosinophils per unit area (2,220 μm^2) was determined by morphometric analysis. *P value, Zileuton/OVA versus OVA. (*B*) BAL fluid was obtained from sham-sensitized and saline-challenged mice (*Saline*; $n = 3$), and OVA-sensitized/challenged mice in the absence (*OVA*; $n = 4$) or presence (*MK-886/OVA*; $n = 3$) of treatment with the FLAP inhibitor MK-886. The number of eosinophils per total lavage fluid collected is shown. *P value, MK-886/OVA versus OVA.

Leukotriene Inhibition Blocks Eosinophil Infiltration and Mucus Accumulation in Airways. 5-Lipoxygenase inhibition by zileuton markedly reduced eosinophil influx into the lung tissue and BAL fluid of OVA-sensitized/challenged mice and also prevented airway mucus release in these animals (Fig. 2 *C*).

Eosinophil Infiltration. By morphometric analysis, the eosinophil influx into the lung interstitium was reduced 82% by zileuton treatment ($P < 0.006$ compared to OVA without zileuton) (Fig. 6 *A*). The FLAP inhibitor MK-886 decreased the number of eosinophils in the BAL fluid by 86% ($P < 0.0004$ compared to OVA without MK-886) (Fig. 6

Figure 5. Mucus release in upper airways in OVA-treated mice. Upper lobe lung sections from OVA-treated mice were stained histochemically for mucus as described in Fig. 4. The airways shown are larger in diameter and have a greater density of smooth muscle cells than the small airways seen in Fig. 4. (*A*) Mucicarmine staining (rose-red) of upper airway mucin is shown. (*B*) Metachromatic toluidine blue staining (red-purple) of nonsulfated acidic mucosubstances and mucin is demonstrated. (*C*) Acidic sulfated mucosubstances, hyaluronic acid, and sialomucins are stained blue by alcian blue. (*D*) Alcian blue/PAS staining (magenta) of neutral and acidic mucosubstances is shown. Bar, 200 μm.

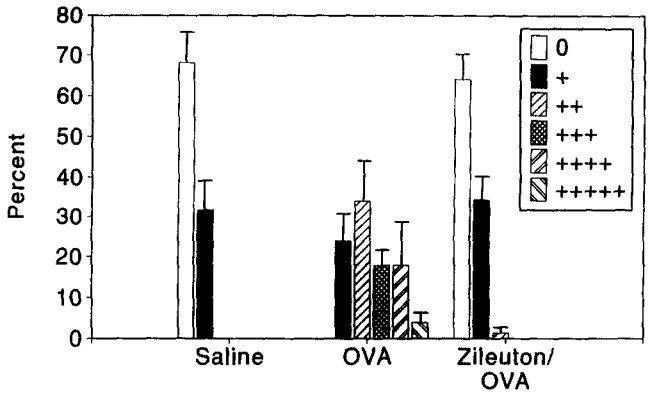


Figure 7. Effect of 5-lipoxygenase inhibition on mucus accumulation in the airways of OVA- treated and control mice. The percent distribution of mucus occlusion of airways from sham-sensitized and saline-challenged mice (*Saline*; $n = 8$), and OVA- sensitized/challenged mice in the absence (*OVA*; $n = 7$) or presence (*Zileuton/OVA*; $n = 7$) of zileuton treatment. Mucus occlusion of airway diameter was assayed morphometrically as follows: 0, no mucus; +, ~10% occlusion; ++, ~30% occlusion; +++, ~50% occlusion; ++++, ~80% occlusion; +++++, 90–100% occlusion. 10 airways randomly distributed throughout the left lung of each mouse were assessed for mucus occlusion morphometrically.

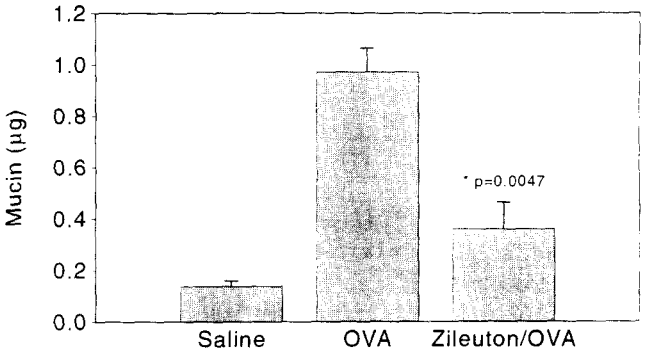


Figure 8. 5-Lipoxygenase inhibition blocks airway mucus release in OVA-treated mice. BAL fluid was obtained from sham-sensitized and saline-challenged mice (Saline; $n = 4$) and OVA-sensitized/challenged mice in the absence (OVA; $n = 4$) or presence (Zileuton/OVA; $n = 4$) of zileuton treatment. BAL fluid (0.05 ml) was blotted onto nitrocellulose with mucin visualized by PAS reaction and quantitated by an image analysis system as described in Materials and Methods. For a standard curve, human respiratory mucin (0.05–0.75 ml, 2 μg/ml) was similarly assayed. * value, Zileuton/OVA versus OVA.

B). The number of eosinophils in the BAL fluid from the OVA-sensitized/challenged mice treated with the FLAP inhibitor ($0.57 \pm 0.11 \times 10^5$, Fig. 6 *B*) was not significantly different from that in either sham-sensitized/saline-challenged ($0.12 \pm 0.02 \times 10^5$, Fig. 6 *B*) or OVA-immunized/saline-challenged ($0.16 \pm 0.03 \times 10^5$, not shown) control mice. Zileuton similarly decreased eosinophils recovered in the BAL fluid of OVA-sensitized/challenged mice by 91% ($P = 0.0128$ compared to OVA without zileuton; data not shown). Vehicle controls (Trappsol™ for zileuton studies, DMSO for MK-886 studies) did not affect the lung eosinophil infiltration observed in OVA sensitized/challenged mice.

Mucus Accumulation. Cross-sections of the upper and lower lobes of the left lung of OVA-treated and control mice were examined by light microscopy for mucus accumulation in the airways. By morphometric analysis, 68% of the airways of control mice treated with saline had no evidence of airway mucus release, and the remainder had only a small mucus layer observed (Fig. 7). In contrast, OVA-immunized/challenged mice had morphologic evidence for widespread mucus plugging of the airways. The majority (74%) of the airways of the OVA-treated mice had at least 30% occlusion of the airway lumen by mucus; 22% of the airways of these mice had 80% or greater mucus occlusion (Fig. 7). When the amount of mucus glycoprotein recovered in the BAL fluid was quantitated (Fig. 8), a sevenfold increase in airway mucin was demonstrated in OVA-treated mice compared to control mice ($P = 0.00001$ OVA versus saline). Zileuton-treatment blocked the airway mucus release in the OVA-treated mice (Figs. 2 *C* and 8). The importance of leukotrienes in airway mucus release was further demonstrated by the ability of LTD_4 administered exogenously by the i.n. route to induce airway mucus release (Fig. 9).

5-Lipoxygenase Inhibition Does Not Alter Allergen-induced Airway Hyperreactivity to Methacholine. Mice receiving i.p. and i.n. OVA had increased airway reactivity compared to control mice receiving saline; the OVA-treated mice required significantly lower concentrations of methacholine to decrease G_L (Fig. 10 *A*) and C_{dyn} (Fig. 10 *B*) than control mice that had been sham-sensitized and challenged with saline (Table 1) or OVA-immunized/saline challenged (not shown). Although leukotriene inhibition blocked airway eosinophil influx and mucus release in OVA-treated mice, zileuton given before OVA on day 25, 26, and 27 did not reduce airway hyperresponsiveness to methacholine in these

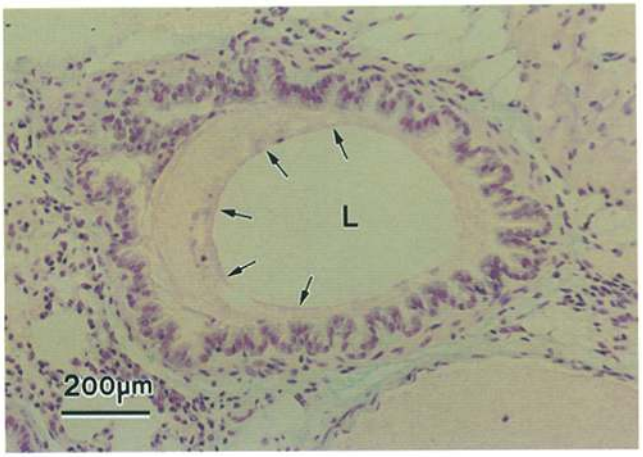


Figure 9. LTD_4 induces airway mucus release. Upper airway tissue was obtained 60 min after i.n. challenge with LTD_4 (2.5 μg) and stained with hematoxylin and eosin. Mucus deposition (*arrows*) in the airway lumen (*L*) is shown. Airway mucus release was not observed in mice receiving i.n. vehicle alone (not shown). Bar, 200 μm.

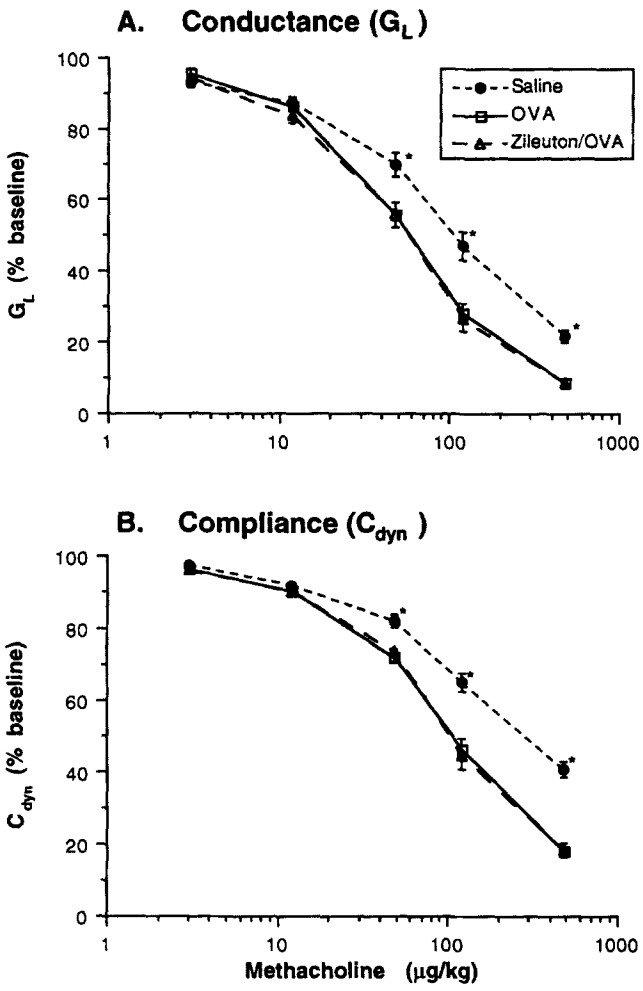


Figure 10. 5-Lipoxygenase inhibition does not alter methacholine-induced airway responsiveness in OVA-treated mice. Airway responsiveness to increasing concentrations of methacholine was determined in sham-sensitized and saline-challenged mice (*Saline*; $n = 19$), and OVA-sensitized/challenged mice in the absence (*OVA*; $n = 18$) or presence (*Zileuton/OVA*; $n = 17$) of zileuton treatment. (*A*) Airway conductance (G_L) and (*B*) dynamic lung compliance (C_{dyn}) were calculated as described in Materials and Methods. The data are mean ± SE. $^*P < 0.05$ by ANOVA, Saline versus the OVA and Zileuton/OVA groups.

animals (Fig. 10, Table 1). No significant difference in basal conductance or compliance was observed among the three groups (Table 1). Zileuton treatment on day 0 and 14 in addition to days 25, 26, and 27 did not alter methacholine-induced lung responses (data not shown). The FLAP inhibitor MK-886 when given 30 min before i.n. OVA-challenge on days 25, 26, and 27 also failed to block the increased airway response to methacholine (data not shown).

Discussion

Our studies demonstrate that leukotrienes are key mediators of allergen-induced airway eosinophil infiltration and mucus release but not the increased airway responsiveness to methacholine occurring in a mouse model of asthma. BALB/c mice given i.p. OVA in alum twice over a 14-d period followed by three i.n. doses of OVA on days 25, 26, and 27 of the protocol developed OVA-specific IgE and histologic and physiologic findings mimicking human asthma. In mice examined 24 h after the last i.n. administration of OVA on day 28, eosinophils were the predominant inflammatory cell in both the lung interstitial tissue and BAL fluid. Mucus occlusion of the airway lumen (lower >upper airways) in the OVA-treated mice was a prominent feature of this late-phase allergen-induced inflammatory process. Further, in vivo pulmonary function tests showed that the OVA-immunized/challenged mice reproducibly developed increased airway responsiveness to intravenous methacholine compared to controls.

In vitro and in vivo studies in animals and humans have suggested that leukotrienes play a role in the pathogenesis of allergic airway inflammation. Nocturnal levels of LTB_4

Table 1. *Pulmonary Mechanics to Methacholine in OVA-treated Mice: Effect of Leukotriene Inhibition*

	Conductance (GL)			Dynamic compliance (C_{dyn})		
		ED_{50} methacholine			ED_{50} methacholine	
Group	Basal	Geometric mean	Log SD	Basal	Geometric mean	Log SD
	ml/s × cm H_2O	*μg/kg*		*ml/s × cm H_2O*	*μg/kg*	
Saline	1.30 ± 0.04	104*	0.29	0.028 ± 0.001	277*	0.29
OVA	1.22 ± 0.03	56	0.20	0.026 ± 0.001	107	0.19
Zileuton/OVA	1.24 ± 0.03	54	0.23	0.026 ± 0.001	111	0.21

Data represent mean ± SE. GL and C_{dyn} were determined in sham-sensitized and saline-challenged mice (*Saline*, $n = 19$), and OVA-sensitized/challenged mice in the absence (*OVA*; $n = 18$) or presence (*Zileuton/OVA*; $n = 18$) of zileuton treatment.
$^*P < 0.05$ vs both other groups (by ANOVA using the protected least significant difference method).

and cysteinyl leukotrienes are elevated in the BAL fluid of patients with nocturnal asthma compared to normal control subjects (35). Increased levels of LTB_4 and LTC_4 are recovered in the BAL fluid (11, 12) and elevated levels of the LTC_4 metabolite LTE_4 are detected in the urine (36) after endobronchial challenge with specific allergen compared to prechallenge levels of these eicosanoids. Similarly, in this mouse model of asthma, we found that levels of LTB_4 and LTC_4 were significantly increased in BAL fluid after airway allergen challenge; this release of leukotrienes was blocked by the specific 5-lipoxygenase inhibitor zileuton.

Various mediators including histamine, platelet-activating factor, prostaglandins, thromboxane A_2, and leukotrienes have been implicated in airway mucus release (37). Cysteinyl leukotrienes C_4 and D_4 stimulate mucus secretion from cat (38), dog (39), and guinea pig (40) trachea and bronchi in vivo and human bronchial explants in vitro (41, 42). We have demonstrated in mice that exogenous LTD_4 induces airway mucus release in vivo. When compared with other mucus secretagogues for inducing release of mucus glycoproteins from cultured human bronchial tissues, the rank order of potency (on a molar basis) is $LTD_4 \geq LTC_4 >$monohydroxyeicosatetraenoic acids $> LTB_4 > PGA_2 = PGD_2 = PGE_1 = PGF_{2\alpha} = PGI_2 >$histamine (37, 43, 44). A striking finding of our study is that inhibition of leukotriene formation prevents allergen-induced mucus plugging of mouse airways in vivo. These results suggest that leukotrienes play a major role in this mucus secretory process.

LTB_4 is recognized as one of the most potent chemoattractants for leukocytes. LTB_4 promotes chemotaxis and chemokinesis of eosinophils from various animal species (45, 46) and humans (7, 47, 48). In patients with nocturnal asthma, the increased LTB_4 levels in nocturnal BAL fluid correlate significantly with the increased number of eosinophils recovered in these samples (35). The cysteinyl leukotrienes also exert potent chemotactic activity for eosinophils. LTD_4 promotes human eosinophil chemotaxis in vitro beginning at 10^{-10} M (7). The specific LTD_4 receptor antagonist MK-571 significantly reduces OVA-induced eosinophil influx into guinea pig conjunctiva in vivo 17 h after allergen challenge (49). Inhalation of LTE_4 in patients with asthma significantly increases the number of eosinophils recovered in the airway lamnia propria 4 h after LTE_4 challenge (6).

In our studies, inhibition of leukotriene release by either a 5-lipoxygenase inhibitor (zileuton) or FLAP inhibitor (MK-886) was found to significantly inhibit eosinophil infiltration into both airway interstitial tissue and the BAL fluid indicating an important role for leukotrienes in mediating allergic airway infiltration by eosinophils, a key feature of asthma. However, these data dissociate the airway eosinophilic influx from the increased airway responsiveness to methacholine. Although leukotriene inhibition markedly reduced the eosinophil infiltration, such inhibition did not affect the bronchial hyperresponsiveness.

Other studies in both rats and mice have also suggested that BAL eosinophilia may be dissociated from airway hyperresponsiveness. When mononuclear cells isolated from peribronchial lymph nodes from OVA-sensitized Brown Norway rats were transferred to naive syngeneic recipients, the recipient rats after OVA aerosol challenge developed late asthma responses (50). In recipients of either sensitized or sham-sensitized mononuclear cells, the number of BAL fluid eosinophils increased at 32 h. A negative correlation between the number of BAL fluid eosinophils and airway responsiveness to methacholine was observed in these studies (50). In OVA-sensitized BALB/c mice, airway hyperresponsiveness to acetylcholine and an influx of eosinophils into the BAL fluid was observed 24 h after the last aerosol challenge with OVA (51). Although treatment with anti-IL-5 mAb during the systemic immunization period decreased BAL fluid eosinophils from 44 to 2%, no effect on the airway response to acetylcholine was seen (51). These results contrast, however, with studies employing IL-5 deficient mice in which eosinophilia was closely correlated with airway reactivity (52). Whereas, airway hyperreactivity to methacholine and airway eosinophilia were absent in aeroallergen-challenged OVA-sensitized IL-5 deficient mice, airway eosinophilia and hyperreactivity were restored with reconstitution of IL-5 production (52). Differences in conclusions regarding the role of eosinophils in airway responsiveness may be specific to the different antigen sensitization/immunization protocols and animal species employed in these studies. Further, many mechanisms other than leukotriene release could potentially mediate the observed pulmonary function changes (e.g., alteration in smooth muscle contractility, neuropeptides, muscarinic receptor defects) (53–55).

Our studies indicate that leukotriene inhibitors have a marked antiinflammatory effect on allergic lung inflammation in an animal model that reproduces many of the features of human asthma. These antiinflammatory actions may prove useful in the therapy of asthma and other inflammatory diseases.

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