

Ragweed Antigen Causes Interleukin-8 Production in Sensitized Dog Trachea

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Antigen challenge in allergic subjects and in sensitized animals causes recruitment of neutrophils as well as eosinophils into the airways. In this study, we asked whether interleukin-8 (IL-8) is involved in the recruitment of neutrophils into the airways after antigen challenge. We administered ragweed antigen to an isolated, superfused tracheal segment of ragweed-sensitized dogs. Local tracheal instillation of the antigen caused an increase in IL-8 concentration and increases in both the number of recruited neutrophils and myeloperoxidase activity (one indicator of neutrophil activation in the recruited cells). The increase in IL-8 concentration was observed earlier than both the neutrophil recruitment and the increase in myeloperoxidase activity in the tracheal superfusate. Superfusate removed from the trachea 8 h after antigen challenge showed marked neutrophil chemotactic activity in a microchemotaxis chamber, and this activity was inhibited (mean, 75.6%) by a blocking antibody to dog IL-8. We conclude that ragweed antigen causes neutrophil recruitment into the airways at least in part because of IL-8 production. **Kaneko T, Massion PP, Hara M, Nadel JA. Ragweed antigen causes interleukin-8 production in sensitized dog trachea.**

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Bronchial asthma is an inflammatory disease, and eosinophils have been suggested to play a major role in the pathogenesis of the disease (1). However, other leukocytes may also play significant roles in certain clinical manifestations. Antigen challenge causes the recruitment of neutrophils as well as eosinophils into the airways in asthmatic subjects (2-4) and in sensitized animals (5-7). Neutrophils have long been recognized to cause tissue damage by releasing oxidants (8) and proteases (9). In addition, neutrophil proteases are extremely potent inducers of secretion in airway submucosal glands (10) and goblet cells (11). Thus, neutrophil proteases might be involved in the mucous plugging of the airways that is reported to occur in asthmatic lungs at post-mortem (12).

Interleukin-8 (IL-8) is a cytokine that is a potent neutrophil chemoattractant (13). This cytokine has been incriminated in neutrophil recruitment into the lungs and airways in various pathologic conditions, including cystic fibrosis, bronchiectasis, and chronic bronchitis (14). The purpose of the present study was to examine the role of IL-8 in antigen-induced neutrophil recruitment into the airways. We examined the effect of local airway administration of ragweed antigen in ragweed-sensitized dogs. We chose this preparation for several reasons: (1) these dogs have previously been shown to possess some of the characteristics of

allergic asthma in humans, including an early bronchoconstrictor response after the inhalation of ragweed aerosol, increased bronchomotor responses to acetylcholine 6 h after antigen challenge, and the recruitment of inflammatory cells (15); (2) the dog possesses a large trachea, and a method exists for isolating a segment of the trachea *in vivo* (16). The isolated tracheal segment can be superfused and antigen can be introduced. Then the tracheal superfusate can be sampled for counting the recruited inflammatory cells and for measuring markers of inflammatory cell activation and mediators of leukocyte recruitment; (3) canine IL-8 has been cloned and expressed (17), so measurement of IL-8 production in the canine airways is possible, and the tracheal superfusate can be removed and assessed for chemotactic activity *in vitro*.

METHODS

In Vivo Studies

Preparation of animals. The experimental protocol followed the published "Guiding Principles in the Care and Use of Animals" of the Council of the American Physiological Society and the NIH "Guide for the Care and Use of Laboratory Animals," and it was approved by the Committee on Animal Research of the University of California San Francisco.

We performed the study in four ragweed-sensitized dogs (mean weight 23.6 ± 2.7 kg) from a colony of inbred ragweed-sensitized dogs (18) and in four nonsensitized dogs (26.2 ± 1.9 kg). The sensitized dogs possess some of the characteristics of allergic human asthma. The responses to inhalation of ragweed aerosol include early bronchoconstriction, increased bronchomotor responsiveness to acetylcholine at 6 h, and the recruitment of inflammatory cells into the airways (15). Superfusion of the isolated trachea with ragweed antigen causes recruitment of inflammatory cells, the release of elastase, and submucosal gland secretion (19).

The dogs were initially anesthetized intravenously with sodium pentobarbital (30 mg/kg) and supplemented later with additional doses of sodium pentobarbital (65 mg/ml) as needed. The dogs were placed on warming pads to maintain body temperature at 37° C. Heart rate was monitored continuously during the experiment and remained unchanged

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after addition of antigen to the superfusate. Intravenous infusion of 0.9% NaCl (50 ml/h) was given to prevent dehydration. After induction of anesthesia, a modified double-balloon endotracheal tube (9.0 mm ID, Hi-Lo Jet Oral; Mallinckrodt, Glens Falls, NY), containing two standard low-pressure inflatable balloons (6 cm apart), was inserted per os into the dog trachea. Then the tube was connected to a constant-volume ventilator (Model 607A; Harvard Apparatus, Dover, MA) set to deliver a tidal volume of 10 ml/kg at a frequency of 20 breaths/min.

Isolated tracheal segment. A segment of the trachea was isolated and superfused as described previously (16). Briefly, this system allows for the continuous superfusion of the tracheal lumen with physiological solutions that contain test substances and permits periodic sampling of the superfusate for analysis of tracheal contents. A modified double-balloon endotracheal tube was inserted per os into the dog trachea, and the balloons were inflated. The wall of the modified endotracheal tube contains two small-bore tubes that open to the surface and permit the circulation of fluid to and from the interballoon tracheal segment. The volume in the tracheal segment was ~10 ml. A polypropylene 20-ml syringe barrel served as a reservoir from which the solution was pumped to and from the interballoon tracheal segment. Initially, the solution (20 ml) was placed in the closed system, which included the isolated tracheal segment, reservoir, and Silastic tubing (Dow Corning Corp., Midland, MI). A peristaltic roller pump (Buchler, Fort Lee, NJ) was used to achieve a constant flow of fluid (14 ml/min) to and from the reservoir. The tracheal superfusate was composed of sterile Hanks' balanced salt solution, which contained Ca^{2+} and Mg^{2+} (HBSS⁺), with added penicillin (final concentration, 100 U/ml) and streptomycin (final concentration, 100 µg/ml) to prevent bacterial growth. Ragweed antigen (short ragweed pollen extract) or vehicle (50% glycerin) was diluted in HBSS⁺ and added to the superfusate at the start of the experiment. The final concentration of short ragweed pollen extract in the superfusate was 42 protein nitrogen units (PNU)/ml. This concentration of ragweed antigen was chosen because one-tenth of this concentration (4.2 PNU/ml) elicited a wheal > 15 mm in diameter after intradermal injection in each dog, and it caused lysozyme secretion into the tracheal segment in preliminary experiments. In control experiments, antigen vehicle (glycerin) was diluted in HBSS⁺ and added to the superfusate in the reservoir, giving a final concentration of 0.05 % (wt/vol). Analysis of the HBSS⁺ and antigen dissolved in HBSS⁺ by the limulus amoebocyte lysate test (Cape Cod, Woods Hole, MA) showed endotoxin contents < 0.03 Endotoxin Units/ml (= 3 pg/ml).

Experimental protocol for dogs in vivo. The experiments were conducted in four ragweed-sensitized dogs and in four nonsensitized dogs. In sensitized dogs, ragweed antigen or vehicle was instilled into the tracheal segment on separate occasions, and each dog served as its own control. In nonsensitized dogs, ragweed antigen was also instilled into the tracheal segment to confirm that the response to ragweed antigen occurs only in sensitized dogs. The dogs were rested for at least 3 wk between exposures. The sequence of experiments in each dog was performed in random order. Samples (1.0 ml) were removed from the reservoir at the beginning of the experiment and then every 2 h for an 8-h period. Removed fluid was replaced by HBSS⁺ containing antibiotics, and results were calculated taking the dilution into account. Cell counts were performed on uncentrifuged and unstained tracheal superfusate with a hemacytometer. A modified Giemsa stain (Diff Quik; American Scientific Products, McGaw Park, IL) was performed on the cytocentrifuged preparations (Cytocentrifuge; Shandon, Pittsburgh, PA).

Measurement of myeloperoxidase activity. Myeloperoxidase activity of the tracheal superfusate was measured using the method of Klebanoff and colleagues (20). Ten to 200 µl of tracheal superfusate were added to a cuvette and brought up to total volume of 1 ml with assay solution. The assay solution was made up daily as follows: H_2O , 26.9 ml; 0.1 M sodium phosphate buffer at pH 7.0, 3.0 ml; 0.1 M H_2O_2 , 0.1 ml; guaiacol, 0.048 ml. The cuvette components are rapidly mixed, the absorbance is determined at 470 nm for 1 min in a recording spectrophotometer (UV-Visible Recording Spectrophotometer, Model UV 160U; Shimadzu Scientific Instruments, Inc., Columbia, MD), and the optical density change per minute is calculated from the initial rate. Myeloperoxidase activity was expressed as mU/ml. One milliunit of enzyme is the amount that consumes 1 nM of H_2O_2 per min.

Measurement of dog IL-8 by ELISA. IL-8 activity of the tracheal superfusate was measured with a sandwich enzyme-linked immunosorbent assay (ELISA) developed for dog IL-8 (17). IL-8 concentrations

were determined by interpolation of their absorbance from a standard curve of recombinant dog IL-8. The lower limit of sensitivity for the assay was 300 pg/ml of dog IL-8.

In Vitro Studies

Neutrophil chemotaxis. Dog neutrophils were isolated from the peripheral blood of two dogs that were not used for *in vivo* studies. EDTA-anticoagulated blood was diluted 1:1 with Ca^{2+} - and Mg^{2+} -free Hanks' balanced salt solution (HBSS⁻), layered on top of Histopaque-1077, and centrifuged at $400 \times g$ for 30 min at 20° C. The monocyte-rich fraction was aspirated and discarded. Contaminated red blood cells were removed from the granulocyte-rich pellet by hypotonic lysis. The neutrophils were washed twice with HBSS⁻ and resuspended in HBSS⁺ with bovine serum albumin (BSA; 1%) and counted in a hemacytometer. The neutrophils were further diluted with HBSS⁺ with 1% BSA to a cell density of 4×10^6 cells/ml. The neutrophil suspension was at least 96% pure as determined by differential cell count of cytocentrifuged preparations stained with Diff Quik, and 99% viable as determined by trypan blue dye exclusion.

The chemotactic activity of the dog tracheal superfusate taken 8 h after antigen challenge from sensitized dogs was assessed in a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, MD) as previously reported (17). Chemotactic activity was assayed by a leading front technique. Neutrophils were allowed to migrate through a nitrocellulose filter of 3 µm pore size for 25 min at 37° C. The results are expressed as net neutrophil migration (migration to sample minus migration to antigen diluted with HBSS⁺). We also assessed the role of IL-8 in the neutrophil chemotactic activity of the tracheal superfusate using a blocking antibody against dog IL-8 (17). The tracheal superfusate was incubated for 30 min at 37° C with the blocking antibody (100 µg/ml), with an antihuman lactoferrin antibody (which served as a control), or with the same volume of buffer (HBSS⁺). As a positive control, we used 3×10^{-8} M recombinant dog IL-8. This concentration was chosen because all samples of the tracheal superfusate 8 h after antigen challenge contained less IL-8 activity (mean, $1.43 \pm 0.23 \times 10^{-8}$ M; range, 0.97 to 2.07×10^{-8} M). The antibodies alone were not chemotactic for neutrophils.

Chemical reagents and drugs. Sodium pentobarbital was purchased from Anthony Products (Arcadia, CA). Sterile HBSS⁺, HBSS⁻, penicillin, and streptomycin were purchased from the Tissue Culture Facility, University of California San Francisco. NaCl solution (0.9%) for infusion was purchased from Travenol Laboratories (Deerfield, IL). Short ragweed (*Ambrosia artemisiifolia*) pollen extract was purchased from Hollister-Stier (Spokane, WA). Guaiacol, BSA, Histopaque-1077, glycerin, and antihuman lactoferrin antibody were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant dog IL-8 and the blocking antibody against dog IL-8 were produced in our laboratory (17).

Statistical Analysis

All data are presented as means $\pm$ SEM. Data from chemotaxis assays were analyzed by Student's paired *t* test. Other data were analyzed using a one-way analysis of variance followed by Student's two-tailed *t* test with the Bonferroni correction for multiple comparisons. Statistical significance was set at *p* < 0.05.

RESULTS

In Vivo Studies

Tracheal superfusion with the control solution caused the recruitment of only a few cells in sensitized dogs, even after 8 h (Figure 1). However, introduction of ragweed antigen in the trachea of sensitized dogs resulted in a time-dependent increase in neutrophils in the superfusate, consisting of $87.9 \pm 8.6\%$ of recruited cells 8 h after antigen challenge (the remaining cells were mostly eosinophils; mean, 10.2%). The number of neutrophils recruited into the superfusate 8 h after antigen challenge (596.4 ± 143.4 cells/µl) was significantly higher than the number after control solution (64.4 ± 21.1 cells/µl). Introduction of ragweed antigen in the trachea of sensitized dogs also caused a time-dependent increase in the myeloperoxidase activity in the superfusate (Figure 2). The myeloperoxidase activity of superfusate 8 h after an-

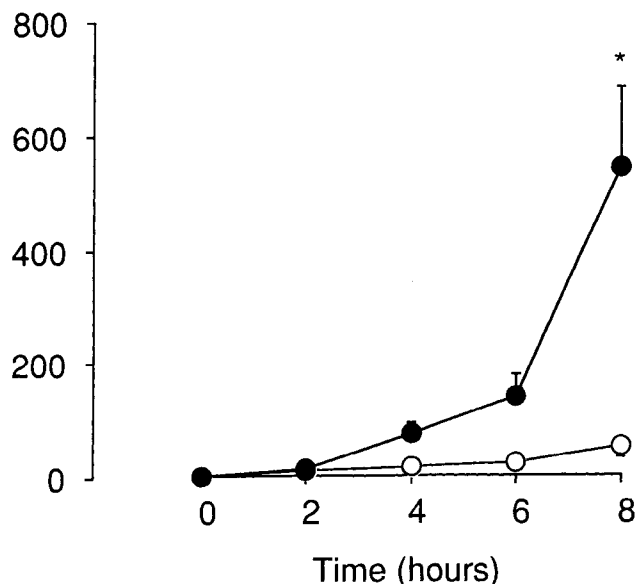
Neutrophils/ μ l

Figure 1. Time course of the effect of ragweed antigen on neutrophil recruitment into the superfused tracheal segment in ragweed-sensitive dogs. Vehicle alone (open circles) had little effect on neutrophil recruitment. Antigen (closed circles) caused a time-dependent increase in neutrophils. Values represent means $\pm$ SEM; $n = 4$. * $p < 0.05$ versus vehicle alone.

tigen challenge (311.9 ± 46.8 mU/ml) was significantly higher than the activity after control solution (12.5 ± 5.2 mU/ml).

Tracheal superfusion with the control solution resulted in the secretion of only a low concentration of IL-8 in sensitized dogs

Myeloperoxidase (mU/ml)

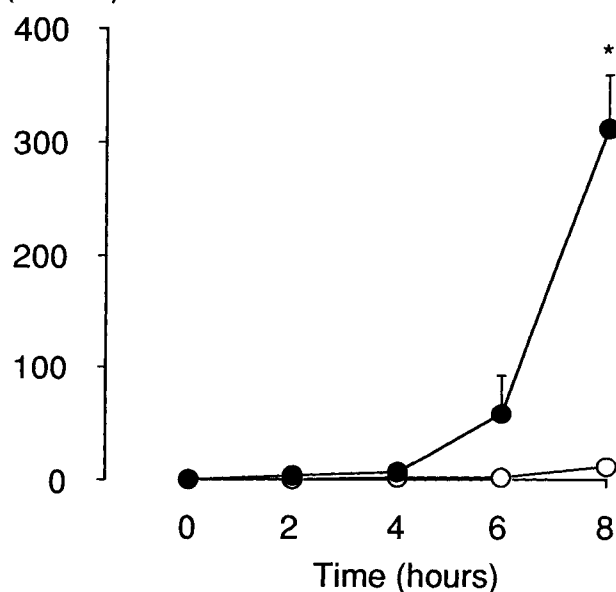


Figure 2. Time course of the effect of ragweed antigen on the myeloperoxidase activity in the superfusate of the by-passed tracheal segment in ragweed-sensitive dogs. Vehicle alone (open circles) caused little effect. Antigen (closed circles) caused a time-dependent increase in the myeloperoxidase activity in the tracheal superfusate. Values represent means $\pm$ SEM; $n = 4$. * $p < 0.05$ versus vehicle alone.

(Figure 3). However, introduction of ragweed antigen in the trachea of sensitized dogs caused a marked time-dependent increase in the concentration of IL-8 in the superfusate. The difference in IL-8 concentration between antigen and control solution reached statistical significance 6 h after introduction, which was earlier than that of both the recruitment of neutrophils and increased myeloperoxidase activity. The IL-8 concentration of the superfusate 8 h after control solution and antigen challenge was 14.3 ± 6.8 and 117.3 ± 20.5 ng/ml, respectively.

In nonsensitized dogs, tracheal superfusion with ragweed antigen did not cause significant effects on the number of recruited neutrophils, myeloperoxidase activity, and IL-8 concentration. The number of recruited neutrophils, myeloperoxidase activity, and IL-8 concentration in the superfusate 8 h after antigen challenge were 44.0 ± 11.0 cells/ μ l, 12.9 ± 8.6 mU/ml, and 9.3 ± 3.1 ng/ml, respectively. These values are similar to those obtained after tracheal superfusion with control solution in sensitized dogs.

In Vitro Studies

Dog tracheal superfusate taken 8 h after antigen challenge from sensitized dogs showed a marked chemotactic activity for dog neutrophils (net neutrophil migration, 43.9 ± 6.5 μ m) (Figure 4). Neutrophil chemotaxis in response to the superfusate was largely inhibited by the blocking antibody against dog IL-8 (100 μ g/ml); the mean percent inhibition of neutrophil chemotaxis by the blocking antibody was 75.6%. Incubation with the control (antihuman lactoferrin) antibody had no effect on neutrophil chemotaxis in response to the superfusate after antigen challenge. In contrast, the positive control (3×10^{-8} M recombinant dog IL-8) showed less net chemotaxis (mean, 33.5 μ m); IL-8-induced chemotaxis was abolished by the blocking antibody against dog IL-8.

Interleukin-8 (ng/ml)

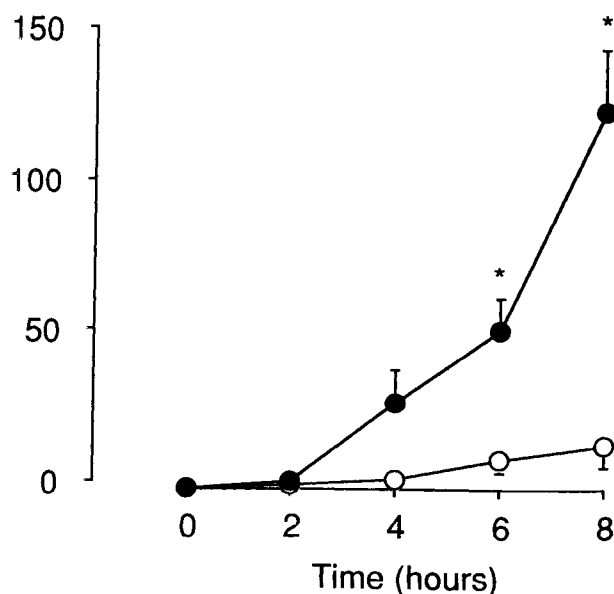


Figure 3. Time course of the effect of ragweed antigen on the concentration of interleukin-8 in the superfusate of the by-passed tracheal segment in ragweed-sensitive dogs. Vehicle alone (open circles) caused little effect. Antigen (closed circles) caused a time-dependent increase in the concentration of interleukin-8 in the tracheal superfusate. Values represent means $\pm$ SEM; $n = 4$. * $p < 0.05$ versus vehicle alone.

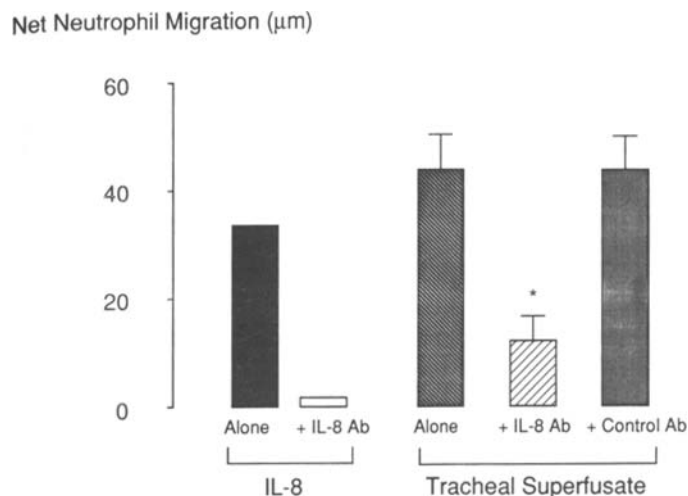


Figure 4. Effect of a blocking antibody (Ab) against dog interleukin-8 (IL-8) on neutrophil chemotactic activity of the tracheal superfusate 8 h after antigen challenge. A blocking antibody to dog interleukin-8 abolished the neutrophil chemotactic activity of 3×10^{-8} M recombinant dog interleukin-8 and inhibited neutrophil chemotactic activity of tracheal superfusate by a mean of 75.6%. Values represent means $\pm$ SEM; $n = 4$. * $p < 0.05$ versus superfusate alone.

DISCUSSION

In the present studies, we show that local antigen challenge in sensitized dogs results in a time-dependent increase in IL-8 concentration in the tracheal superfusate. Eight hours after antigen challenge, the tracheal superfusate contained an IL-8 concentration (mean, 117.3 ng/ml = 1.40×10^{-8} M) in the range that causes maximal neutrophil recruitment in the dog trachea (21). Furthermore, 8 h after the intratracheal administration of antigen, superfusate removed from the trachea caused marked stimulation of neutrophil chemotactic activity in a microchemotaxis chamber, and this activity was largely inhibited by a blocking antibody to dog IL-8, thus implicating IL-8 in the recruitment of neutrophils after antigen challenge in dogs.

The sources of the early induction of IL-8 in the trachea are likely to be the epithelial and gland duct cells, which have been reported to express IL-8 in response to TNF α and to products of *Pseudomonas aeruginosa* bacteria (22). The recruited neutrophils themselves are sources of additional IL-8 (22–24). Thus, recruited neutrophils may be involved in recruiting more neutrophils into the airways as a positive feedback by releasing IL-8. Furthermore, eosinophils (25) and mast cells (26) are reported to release IL-8, and they may thus contribute directly to the IL-8 production in the airways after antigen challenge.

The mechanisms by which antigen causes IL-8 production in the airways are not known. Endotoxin has been suggested as the cause of neutrophil recruitment into the airways after antigen challenge (27), but this is not likely in our study for the following reasons. First, our antigen solution contained less than the detection limit of endotoxin (< 0.03 Endotoxin Units/ml = 3 pg/ml). Second, endotoxin in concentrations as high as 10 μ g/ml does not cause neutrophil recruitment into the canine tracheal segment (22). Third, previous studies have reported that endotoxin does not cause IL-8 production in cultured airway epithelial cells (28, 29).

Other more likely causes of IL-8 production after antigen in the airways are IL-1 β and TNF α , two cytokines that have been reported to be potent stimuli of IL-8 induction in airway epithelial cells (28) and in neutrophils (23). In support of this possibil-

ity, increased expression of TNF α and IL-1 β is reported in the airways of symptomatic asthmatics (30, 31). In addition, granulocyte-macrophage colony-stimulating factor (GM-CSF) may be important as a potent stimulus for IL-8 production in neutrophils (24). Increased expression of GM-CSF mRNA has also been reported in asthmatic airways (32, 33).

Previous studies have shown that introduction of antigen in sensitized animals causes neutrophil as well as eosinophil recruitment into the airways (5–7). Similarly, in asthmatic subjects, introduction of antigen results in both neutrophil and eosinophil recruitment into the airways (2–4). Because antigen causes neutrophil recruitment into the airways, it is important to examine its relevance to the pathogenesis of asthma after the inhalation of antigen. Of particular potential clinical importance is the effect of neutrophils on mucus secretion in airway submucosal glands and goblet cells: neutrophil elastase and, to a lesser extent, cathepsin G are powerful secretagogues (10). In our dog model, superfusion of the isolated trachea with ragweed antigen caused recruitment of inflammatory cells, the release of elastase, and submucosal gland secretion (19). Asthmatic subjects have metaplasia of the airway epithelium to goblet cells and hyperplasia of submucosal gland cells (34). Thus, we speculate that neutrophil recruitment into the airway lumen after antigen could cause goblet cell degranulation in peripheral airways and submucosal gland degranulation in large airways. We suggest that such an event could contribute to the mucous plugging of the airways in acute asthmatic attacks described post-mortem in patients dying with asthma (12).

In the present studies, a blocking antibody to dog IL-8 markedly decreased but did not totally abolish the chemotactic activity present in the tracheal superfusate after antigen challenge, suggesting that other chemoattractants may play a role in neutrophil recruitment after antigen challenge. One of the candidates is leukotriene B $_4$, which has been implicated in the recruitment of neutrophils after segmental antigen challenge in asthmatic subjects (35).

In summary, we have shown that antigen challenge results in increased IL-8 concentration, neutrophil recruitment, and increased myeloperoxidase activity in canine tracheal superfusate in sensitized dogs. The tracheal superfusate 8 h after antigen challenge caused marked neutrophil chemotactic activity *in vitro*, an effect that was largely inhibited by a blocking antibody to dog IL-8. We conclude that IL-8 may be involved in the recruitment of neutrophils after antigen challenge.

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