

Soot and House Dust Mite Allergen Cause Eosinophilic Laryngitis in an Animal Model

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Objectives/Hypothesis: Chronic laryngitis (CL) is common and costly. One of the most common causes of CL is thought to be laryngopharyngeal reflux, although a significant percentage of individuals fail to get better with acid suppressive therapy. The role of other potential causes of CL such as allergy and environmental pollution has not been thoroughly investigated.

Purpose: To evaluate the association between iron soot, house dust mite allergen (HDMA), and CL in an established animal model.

Methods: Twenty-four guinea pigs were separated into four 6-week exposure groups: 1) saline (allergen control) + filtered air (pollution control); 2) HDMA (*Dermatophyoides farinae*) + filtered air; 3) saline + combustion particulates; or 4) HDMA + combustion particulates. The primary outcome measure was mean eosinophil profile (MEP) in glottic, subglottic, and trachea epithelium and submucosa.

Results: The combination of iron soot and HDMA caused eosinophilia (elevated MEP) in the glottic ($P < 0.06$), subglottic ($P < 0.05$), and trachea ($P < 0.05$) submucosa and epithelium ($P < 0.05$).

Conclusion: The combination of HDMA and iron soot resulted in laryngeal eosinophilia in an established guinea pig model of CL. The data support the notion that factors other than reflux may cause CL. Further investigation into eosinophilic laryngitis as a distinct clinical entity caused by exposure to environmental allergen and pollution is warranted.

Key Words: Chronic laryngitis, laryngitis, laryngopharyngeal reflux, LPR, allergy, house dust mite antigen, HDMA, combustion particles, pollution, iron soot, soot, environment.

Level of Evidence: N/A.

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INTRODUCTION

Chronic laryngitis (CL) is common and costly. Symptoms of CL include hoarseness, excessive throat mucus, throat clearing and pain, airway irritability, laryngospasm, the sensation of postnasal drip, cough, dysphagia, and globus.¹⁻⁹ Symptoms of CL are responsible for 4% to 10% of all otolaryngology outpatient visits.¹ As much as 35% of the general population may have symptoms suggestive of CL, and endoscopic signs of occult laryngeal inflammation may exist in 64%.¹⁰ One of the most common causes of CL is thought to be laryngopharyngeal reflux (LPR). Regurgitated gastric contents into

the larynx may cause CL symptoms and laryngeal inflammation, granuloma formation, and possibly laryngeal carcinoma.^{4,7,11-13} Although CL has been attributed to reflux in a substantial proportion of patients, a significant percentage of persons with symptoms of laryngopharyngeal reflux (LPR) fail to get better with reflux therapy, and many individuals develop chronic laryngitis without a clear etiology. The role of other potential causes of CL, such as allergy and environmental pollution, has not been adequately studied.

Laryngopharyngitis was documented at unprecedented levels in clinical studies of World Trade Center (WTC) disaster and recovery workers that were chronically exposed to high levels of environmental particulates and other pollutants.¹⁴ Atopy was found to be a risk factor for WTC-related upper and lower airway disease. This and other recent findings have raised the question of how many patients with allergy- and pollution-related laryngitis are being misdiagnosed with LPR and highlight the concept that the larynx is an important immunological site, functioning as a common gateway between the gastrointestinal and respiratory tracts.

House dust mite allergen (HDMA) is ubiquitous in our environment and homes (carpets, drapes, blankets, pillows, etc.). We have previously found that this allergen produced laryngeal eosinophilia and caused alterations in mucus production in guinea pigs.¹⁵ Carbon

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Exposure Time Course

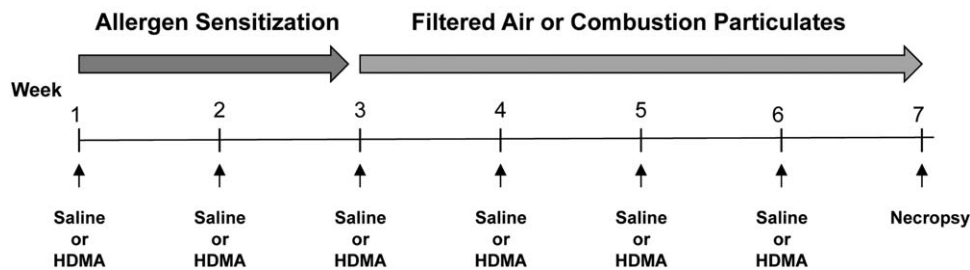


Fig. 1. Time course of exposures for 6-week experiment. At 3 weeks of age, guinea pigs in the HDMA groups were challenged with the allergen once a week via intranasal instillation. Control animals received saline instillation. After 2 weeks of allergen sensitization, animals were exposed to either iron soot combustion particles or filtered air for 6 hours/day for 4 days per week for 4 consecutive weeks. HDMA = house dust mite allergen.

black soot is a worldwide airborne pollutant produced as a byproduct of internal combustion engines, boilers, waste incineration, fires, cooking, candles, and so forth, which has been found to exacerbate respiratory disease. The effect of this environmental pollution on the larynx has not been systematically evaluated. The purpose of this study was to evaluate the association between soot, HDMA exposure, and laryngitis.

MATERIALS AND METHODS

All protocols were approved by the Institutional Animal Care and Use Committee of the University of California, Davis, in compliance with the American Association of Accreditation of Laboratory Animal Care (AAALAC).

Animals

Twenty-four male juvenile Hartley guinea pigs (2 weeks old) were received from Charles River Laboratories (Hollister, CA). Animals were housed at the UC Davis Center for Health and the Environment (CHE) in pairs in large tub containers to allow for socialization and interaction. They were kept on a 12-hour light/dark cycle and had free access to food and water. After acclimation for 1 week, all animals were randomly assigned to one of the four following treatment groups ($n = 6/\text{group}$): 1) saline (allergen control) + filtered air (pollution control); 2) house dust mite allergen (*Dermatophyoides farina* – HDMA) + filtered air; 3) saline + combustion particulates; or 4) HDMA + combustion particulates.

Exposures and Treatments

All exposures and treatments occurred at the inhalation facility of the CHE. Beginning at 3 weeks of age, guinea pigs in the HDMA groups were challenged once a week (on Monday) for a total of 6 consecutive weeks to intranasal instillation of HDMA (150 μg HDMA delivered in 150 μl of saline [75 μl per nostril]). Animals not exposed to HDMA were given saline intranasal instillations. After 2 weeks of allergen sensitization, animals were exposed to iron soot combustion particulates or air passed through a HEPA filter for 6 hours a day, 4 days per week (Tuesday–Friday) for 4 consecutive weeks. During the exposure period, pairs of animals in the same treatment group were placed in wire-top polycarbonate plastic cages in whole-body exposure chambers. Figure 1 depicts the exposure protocol.

HDMA

HDMA stock (Greer Laboratory, Lenoir, NC) was depleted of endotoxin prior to suspension in saline with a ReductEtox

column (Sterogene Bioseparations, Carlsbad, CA), which reduced the level of *lipopolysaccharides* in the HDMA solution to < 0.1 EU/mg protein, as determined with a QCL-1000 kit (Cambrex, Walkersville, MD). This was done to eliminate inflammation due to bacterial wall contamination.

Iron and Soot Combustion Particulate

A diffusion flame system was used to generate an aerosol of soot and Fe oxide. The fuel was a mixture of acetylene and ethylene. Iron was introduced by passing argon over liquid Fe pentacarbonyl. The aerosol emission from the flame was diluted in a secondary chamber with air so that the animals were exposed to ultrafine particles (50–75 nm core particle diameter) at a concentration of 150 to 200 $\mu\text{g}/\text{m}^3$ for the duration of the daily exposure period. Particulate size was analyzed using a size mobility particle sizer (SMPS) system. Particle concentration evaluated gravimetrically using a glass-lined paper filter.

Necropsy and Tissue Preparation

Animals were euthanized 18 hours from the completion of exposure with a combination of isoflurane and pentobarbital prior to the removal of tissue. This is a standard protocol approved by AAALAC for euthanasia of guinea pigs and consistent with recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. The larynx, trachea, and lungs were removed en bloc. The larynx was excised and split longitudinally (Fig. 2). One-half of the larynx was frozen in liquid nitrogen and stored at -80°C until cytokine mRNA analysis. The other half was fixed with 4% paraformaldehyde for 1 hour, embedded in paraffin, and cut to 5- μm thick serial sections mounted on glass slides. This embedding approach has been useful to identify the glottic and subglottic regions of the larynx and the trachea for inflammatory, cellular, and structural examination.

Histochemistry

Paraformaldehyde-fixed sections were stained with the following: 1) hematoxylin and eosin to evaluate the morphology of the larynx; 2) alcian blue-periodic acid Schiff (AB-PAS) to identify epithelial mucosubstances; and 3) combined eosinophil and mast cell stain for identification of eosinophils and mast cells.

Design-Based Stereology

Image analysis software (Image J) was used to obtain quantitative measurements in paraformaldehyde-fixed sections containing the glottis, subglottis, and trachea. For each animal, randomly selected fields of the epithelium and submucosa were imaged at $10\times$ and analyzed. In addition, a mucin index was

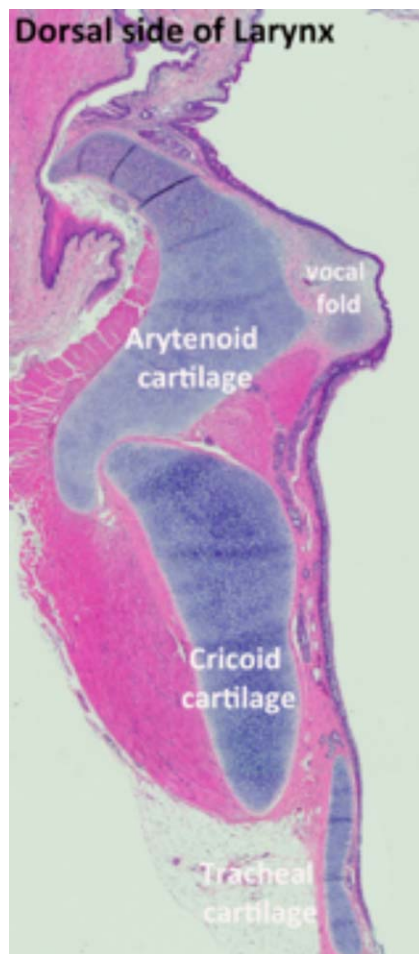


Fig. 2. Sagittal section of the excised larynx and trachea. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

assigned to each field by assessing the percentage of AB-PAS staining cells, normalized to both the length of the underlying basal lamina and to the total airway epithelial volume.

Statistical Analysis

A power analysis determined that at an alpha of 0.05, six animals/group would achieve a power of 0.80 to detect a 30% change in the primary outcome variable (mean eosinophil profile). Differences between control and treatment groups were evaluated with the analysis of variance with post-hoc Tukey-Kramer Honestly Significant Difference test. Significance was considered at $P < 0.05$.

RESULTS

Mean Eosinophil Profile

Glottic Epithelium and Submucosa. The mean eosinophil profile (MEP) of the glottic epithelium and submucosa for the Soot/HDMA group was marginally greater than the MEP in the air/PBS and air/HDMA control groups ($P < 0.06$) (Fig. 4a).

Subglottic Epithelium and Submucosa. The MEP of the subglottic epithelium and submucosa for the soot/HDMA group was significantly greater than the

MEP in the air/phosphate-buffered saline (PBS) and soot/PBS control groups ($P < 0.05$) (Fig. 4b).

Trachea Epithelium and Submucosa. The MEP of the trachea epithelium and submucosa for the soot/HDMA was significantly greater than the MEP in the air/PBS, air/HDMA, and soot/PBS control groups ($P < 0.05$) (Fig. 3 and Fig. 4c).

Mucin Index

There was no difference in the mucin index between the four treatment groups (Table I) ($P > 0.05$).

Epithelium Volume

There was no difference in the epithelium volume between the four treatment groups ($P > 0.05$).

DISCUSSION

The concept of the unified airway suggests that the upper and lower aerodigestive tracts function as a codependent physiologic mechanism.^{16–19} According to this model, the presence and severity of pathologic processes within the upper and lower aerodigestive tracts are closely linked; disease in one component, such as the nose or sinus cavities, is likely to exacerbate disease in another region, such as the larynx, esophagus, or trachea. It is well recognized that environmental pollution and allergens affect the nose and lungs. Work from our lab has previously revealed that repeated exposure to soot and iron particles cause oxidative stress and decreased cell viability throughout the lower respiratory tract.^{20,21} The purpose of the current investigation was to evaluate the effect of environmental allergens and pollution on the larynx. Establishing a link between pollution, sensitized allergens, and laryngeal pathology may provide valuable insight into the pathogenesis of chronic laryngitis, lend further support to the concept of a unified aerodigestive tract, and lead to innovative new therapies.

We have previously reported laryngeal eosinophilia in response to HDMA exposure.²² Guinea pigs exposed to intranasal HDMA for 9 consecutive weeks displayed over twice as much eosinophilia in the supraglottis as animals

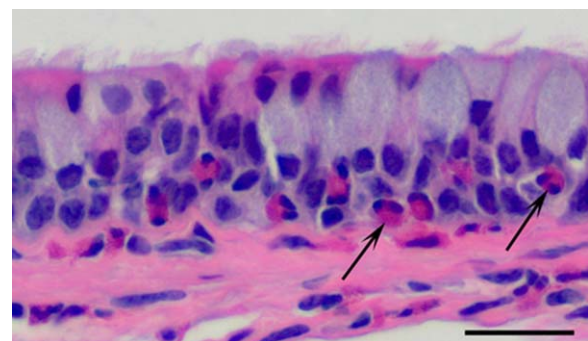


Fig. 3. Eosinophilia in the tracheal epithelium of animals exposed to soot/house dust mite allergen. Arrows point to two of the eosinophils present (scale bar = 25 μ m). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

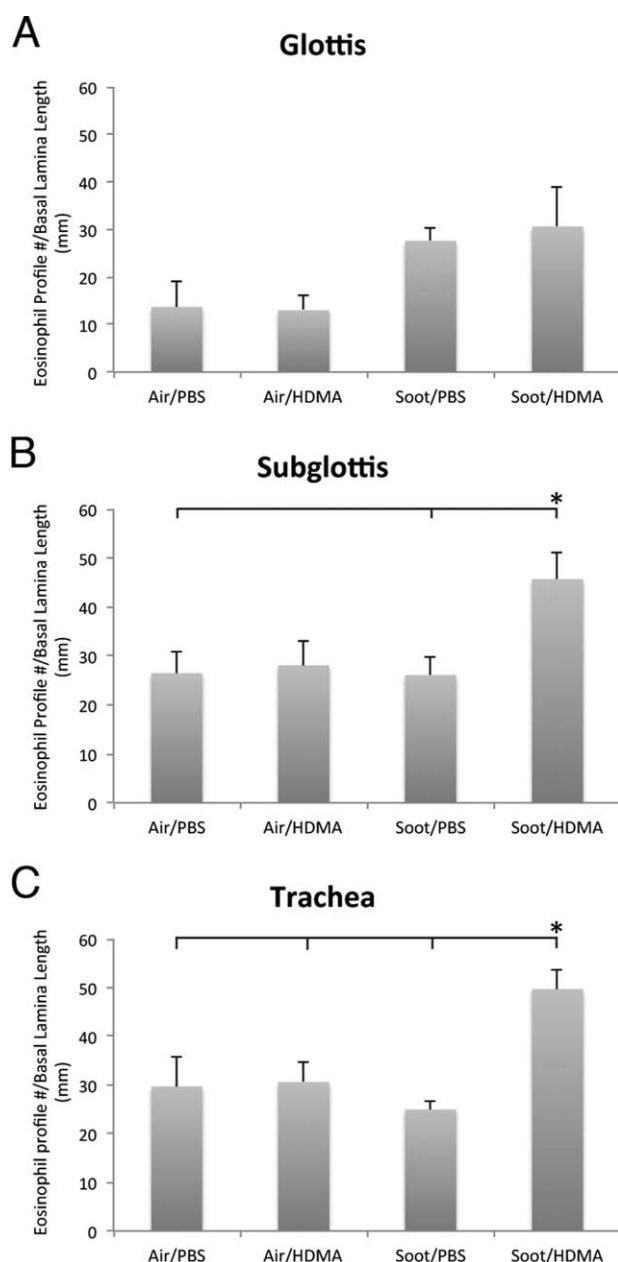


Fig. 4. Mean eosinophil profile in the epithelial and submucosal compartments of the (A) glottis, (B) subglottis, and (C) trachea, respectively. Brackets show significant differences between exposures (*, $P < 0.05$). HDMA = house dust mite allergen. PBS = phosphate-buffered saline.

exposed to intranasal saline. The results of the current investigation expand on this work and suggest that the combination of iron soot and HDMA cause eosinophilia in the glottic, subglottic, and tracheal epithelium and submucosa ($P < 0.05$). In contrast to our previous work, the current investigation did not show an increase in eosinophilia from HDMA exposure alone. The shorter exposure time (6 weeks) or the absence of supraglottic tissue for analysis may account for this discrepancy. Nonetheless, these results confirm the work of previous investigators and provide evidence to support the hypothesis that the environment can cause laryngitis.

Ishii et al. (1997) observed that rats exposed to cedar allergen and particulate matter developed nasal and laryngeal eosinophilia.^{23,24} Leonard et al. (1995) identified histologic evidence of CL in bonnet monkeys exposed to daily ozone for 3 months.²⁵ Translating this work to humans, Dworkin et al.²⁷ induced laryngitis in individuals exposed to HDMA and Naito et al.²⁴ found that individuals with allergy and pollution exposure had more throat symptoms than patients with only allergies.²⁴ Similarly, Randhawa et al. found that patients with a greater number of allergies had worse subjective report of dysphonia.²⁶ These studies and others begin to establish a link between the environment and laryngitis.^{27–29}

Eosinophilia is a hallmark of epithelial inflammation. Generalized eosinophilia is a feature of asthma and allergic rhinitis.^{16,17,30–33} Eosinophilia is also seen in the esophagus as a response to allergy and gastroesophageal reflux. The notion that aerodigestive eosinophilia can cause patient symptoms has also been recognized. Eosinophilic bronchitis (EB) is a clinical condition defined as sputum eosinophilia greater than 2.5%. Macrophages predominate in sputum and eosinophils account for less than 2% of cells in normal individuals. Eosinophilic bronchitis has been implicated in up to 30% of individuals suffering from chronic cough^{34–36}. Similarly, eosinophilic esophagitis (EoE) represents a chronic, immune antigen-mediated esophageal disease characterized by symptoms of dysphagia related to esophageal dysfunction and histologically by eosinophil-predominant inflammation.³⁷

Because EB and EoE are distinct clinical entities, it is reasonable to hypothesize that eosinophilic laryngitis may itself represent a pathologic condition. The identification of laryngeal eosinophilia in response to a challenge with HDMA and iron soot in the current investigation supports the notion that environmental allergens and environmental pollution may act synergistically to contribute to the development of CL. These findings may explain why many patients with a clinical diagnosis of LPR do not respond to reflux therapy. Eosinophilia in other organ systems responds well to treatment with steroids. Both EB and EoE are treated with inhaled and topical steroids, respectively. Topically swallowed medication would not be an appropriate method to deliver steroids to the larynx. Similarly, contemporary inhalers are formulated to deposit steroids to the distal airways. The larynx, however, is accessible for

TABLE I.
Mean Stored Mucosubstance (Mucin) in the Glottis, Subglottis, and Trachea in Each of the Four Treatment Groups

Group	Glottis	Subglottis	Trachea
Air/PBS	22.4 (± 7.3)	33.0 (± 5.3)	25.3 (± 4.2)
Air/HDMA	21.1 (± 8.1)	34.9 (± 4.8)	29.7 (± 4.6)
Soot/PBS	30.6 (± 6.6)	33.7 (± 4.8)	25.6 (± 4.2)
Soot/HDMA	28.5 (± 7.2)	33.1 (± 4.8)	21.4 (± 4.6)

HDMA = house dust mite allergen.

office injection, and we are currently evaluating the effect of intralaryngeal steroid injection for patients with suspected environmentally induced eosinophilic laryngitis unresponsive to reflux therapy. Our technique involves injecting 0.5 cc of triamcinolone 40 mg/cc into each false vocal fold through a thyrohyoid approach (Bristol-Myers Squibb, NY, NY).

This study is not without limitations. This animal model is a surrogate for the human condition. The findings of laryngeal eosinophilia in the guinea pig in response to allergen and pollutant challenge does not equate to a clinical entity in humans, and the results of this investigation must be interpreted with caution before generalizing to human chronic laryngitis. These results should also be replicated by other labs before definitive conclusions are drawn and we acknowledge that proceeding to a clinical trial evaluating the efficacy of intralaryngeal steroid injections for recalcitrant chronic laryngitis may be premature. Nonetheless, these findings suggest that environmental pollution and allergy may work synergistically to cause laryngeal inflammation and support the notion that factors other than reflux may cause chronic laryngitis. Further research is warranted.

CONCLUSION

The combination of HDMA and iron soot resulted in laryngeal eosinophilia in an established guinea pig model of chronic laryngitis. The data support the notion that factors other than reflux may cause chronic laryngitis. Further investigation into eosinophilic laryngitis as a distinct clinical entity caused by exposure to environmental allergen and pollution is warranted.

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