

The effects of allergens and tobacco smoke on the laryngeal mucosa of guinea pigs

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OBJECTIVE: The purpose of this study was to investigate the effects of environmental tobacco smoke (ETS) and house dust mite allergen (HDMA) on the laryngeal mucosa of guinea pigs.

STUDY DESIGN: Prospective, controlled animal study.

SUBJECTS AND METHODS: Sixteen juvenile guinea pigs were randomly assigned to one of four treatment groups: (1) filtered air, (2) HDMA, (3) ETS, and (4) ETS and HDMA. The mucosal response of retrieved larynges was measured by eosinophil grade and stored mucin index.

RESULTS: HDMA exposure increased eosinophil grade in the supraglottis. The mean eosinophil grade in the supraglottis was $1.25 (\pm 1.26)$ in the filtered air group. The grade increased to $3.60 (\pm 0.89)$ in the HDMA group ($P = 0.021$) and $3.00 (\pm 1.16)$ in the ETS/HDMA group ($P = 0.078$). HDMA and ETS exposure was associated with increased mucin index in the subglottis. The grade increased from $1.50 (\pm 1.73)$ in the control group to $3.25 (\pm 0.50)$ in the ETS, $2.80 (\pm 1.64)$ in the HDMA, and $3.25 (\pm 0.50)$ in the ETS/HDMA group ($P < 0.05$).

CONCLUSION: Exposure to HDMA was associated with eosinophilia in the supraglottis. Exposure to HDMA and ETS was associated with elevated mucin in the subglottis. These results provide further evidence that ETS and inhaled allergens may play a role in the development of chronic laryngitis.

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Environmental factors such as secondhand smoke and allergens are known to play an important role in the development of asthma. However, the effects of these challenges on the larynx have been neglected.

A third of the adult population experiences a voice disorder during their lifetime and 7% report a current voice problem.¹ In older persons, the overall prevalence of dysphonia is 20% with half reporting significant impairment of quality of life.² Although the majority of episodes are transient, a substantial proportion becomes chronic without a clear etiology.

Recent evidence suggests that the upper and lower respiratory tract function as one integrated unit. This has given rise to the paradigm of the unified airway. Both epidemiologic and pathophysiologic associations have been described between allergic rhinitis, chronic rhinosinusitis, and asthma.³ Despite a rapidly increasing understanding of the unified airway, the substantial component of the tract occupied by the larynx has been relatively neglected.

The larynx is positioned at the divergence of the respiratory and digestive tracts. It is ideally located as a filter for the airways because it is situated in an area of high turbulent airflow, leading to deposition of incident particles.⁴ There have been only three previous studies to our knowledge looking at the effects of pollutants on the larynx. Leonard et al^{5,6} observed inflammatory changes in the laryngeal epithelium with exposure to environmental ozone in a primate model. Naito et al⁷ found no change in laryngeal eosinophils induced by intranasal exposure to cedar pollen in a rodent model. Although these results are intriguing, further investigation is needed to elaborate both the immediate and long-term effects of various levels of irritant exposure on laryngeal mucosa.

We hypothesized that environmental irritants contribute to the development of laryngeal mucosal disease, including inflammation and neoplasia. The aim of this study was to investigate the effects of environmental tobacco smoke and allergens on laryngeal mucosa in a guinea pig model.

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.

Protocol

Sixteen Hartley guinea pigs, 2 weeks of age, were received from Charles River Laboratories. One week after acclima-

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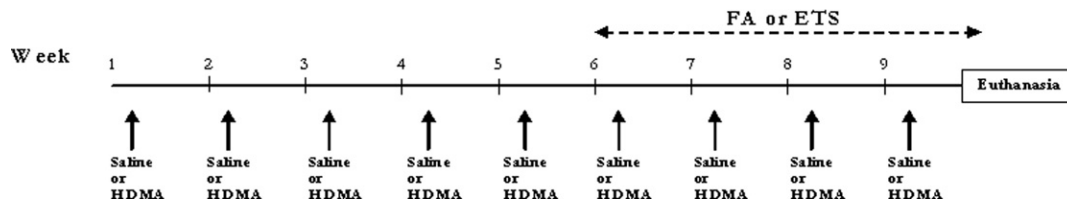


Figure 1 A diagram of the guinea pig treatment course.

tion, all animals were randomly assigned to one of four treatment groups ($n = 4/\text{group}$): (1) filtered air (FA), (2) house dust mite antigen (HDMA); *Dermatophagoides farinae* (endotoxin free) (Greer Laboratories, Inc, Lenoir, NC) (3) ETS, and (4) ETS and HDMA. All animals were maintained as pairs in wire-top polycarbonate plastic cages during each daily exposure period. During nonexposure periods, the guinea pigs were housed (by treatment group) in large tub containers to allow for socialization and interaction.

Exposures and Treatments

Beginning at 3 weeks of age, guinea pigs in the HDMA groups were challenged once a week for a total of 9 consecutive weeks to intranasal instillation of either 150 μg of HDMA delivered in 150 μL of saline (75 μL per nostril). Guinea pigs not exposed to HDMA received intranasal instillation of 150 μL of normal saline. ETS exposure began at 8 weeks of age. ETS exposure to 1 mg/m^3 of total suspended smoke particulate occurred 4 days a week 6 hours a day for 4 weeks. Groups not exposed to ETS were exposed to FA only (Fig 1).

ETS exposure conditions were generated by the burning of 2R4F research cigarettes (University of Kentucky, Lexington, KY) using a Teague E10 smoking machine (Teague Enterprises, Woodland, CA). Each cigarette was burned using a 35-mL puff volume of a 2-second duration once each minute for up to 8 minutes. The smoke from the smoldering end of each cigarette was transported to a chamber where the smoke was aged and diluted over a period of 2 to 4 minutes. Diluted smoke from this chamber was introduced into chambers holding animals. Exposure conditions to ETS were measured according to previously described protocols.⁸ Particulate concentration was determined every 30 minutes using a piezo balance. Carbon monoxide was also sampled every 30

minutes using a carbon monoxide analyzer. Nicotine was determined by gas chromatography. An hour-long sample of the smoke was tested weekly to determine nicotine concentration (Table 1).

HDMA or saline-sham intranasal instillations for all guinea pigs were performed each Monday morning, whereas exposure to either FA or ETS was performed Tuesday through Friday for a total of 6 h/d. Animals exposed to FA alone or in combination were in a chamber identical to the smoke exposure chamber, but, instead of receiving ETS, they received air passed through a HEPA filter.

Euthanasia of animals was performed within 1 to 2 hours of the completion of exposure to ETS (at 12 weeks of age). At the time of euthanasia, the larynges were excised and split longitudinally, fixed in 1% paraformaldehyde, and embedded in paraffin. Serial tissue sections 5- μm thick were prepared. Sections were mounted on clean glass slides and stained with hematoxylin and eosin (H&E), Alcian Blue-Periodic Acid Schiff (PAS), and combined eosinophil mast cell stain from American MasterTech (Lodi, CA).

Histopathology

For the purpose of analysis, 10 representative fields from each subsite (supraglottis, glottis, and subglottis) were examined for each animal. Semiquantitative evaluation was performed by an examiner who was blinded to the treatment group. The eosinophil count was determined by counting the number of eosinophils per 10 high-power field, and a grade was determined (Table 2). A mucin index was assigned to each field by assessing the percentage of PAS staining of the airway epithelium (Table 3).⁹

Statistical Analysis

All data were coded and recorded into SPSS 16.0 for the Macintosh (SPSS Inc, Chicago, IL). Sample-size calcula-

Table 1
ETS exposure data

	Mean ($\pm$ SD)
Relative humidity (%)	23.9 ($\pm$ 8.6)
Temperature ($^{\circ}\text{F}$)	72 ($\pm$ 1)
Carbon monoxide (ppm)	3.0 ($\pm$ 0.5)
Nicotine ($\mu\text{g}/\text{m}^3$)	167 ($\pm$ 24)
Total suspended particulate (mg/m^3)	1.02 ($\pm$ 0.04)

Table 2
Eosinophil grading system

Grade	Number of eosinophils/10 HPF
0	0-4
1	5-9
2	10-14
3	15-19
4	>19

Table 3
Histologic stored mucosubstance (mucin) index

Grade	% airway epithelium with PAS staining
0	0 (no PAS staining)
1	<26
2	26-50
3	51-75
4	>75

tions determined that a sample size of four animals in each group had sufficient power (80%) to ascertain a two-point difference in the eosinophil and mucin indices (assuming a two-sided test with a standard deviation of 1.5 and $\alpha = 0.05$). A one-way analysis of variance was used to compare global differences between exposure groups for each of the laryngeal subsites (supraglottis/glottis/subglottis). The Mann-Whitney *U* (MWU) test was used to compare differences between individual exposure groups. Odds ratios with 95% confidence intervals were used to estimate risk for specific exposure groups. A significance level of 0.05 with the appropriate Bonferroni correction was used for all post hoc analyses.

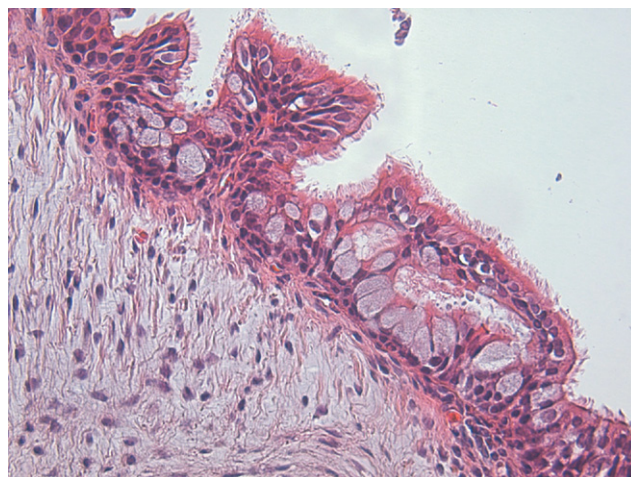
RESULTS

Eosinophils

Table 4 displays the mean eosinophil grade stratified by exposure category. Exposure to HDMA and/or ETS did not appear to have any effect on the eosinophil count in the glottis or subglottic larynx (one-way analysis of variance [ANOVA] *P* value = 0.63 and 0.50, respectively). Exposure to HDMA was associated with an elevated eosinophil grade in the supraglottic larynx (one-way ANOVA *P* value = 0.01). The mean eosinophil grade in the supraglottic larynx was 1.25 (± 1.26) in the control group (FA). Exposure to ETS alone did not appear to have any effect on eosinophil grade in the supraglottis (mean grade = 1.00 [± 1.41]; MWU *P* value = 0.69). However, exposure to HDMA with and without ETS exposure, increased the eosinophil count in the supraglottis in comparison to the control group. The mean eosinophil grade in the supraglottis increased to 3.6 (± 0.89) in the HDMA and 3.00 (± 1.16) in the ETS/HDMA groups (MWU *P* value = 0.03 and 0.07, respectively).

Table 4
Mean eosinophil grade ($\pm$ SD) by exposure group

Group	Supraglottis	Glottis	Subglottis
Control (FA)	1.25 (± 1.26)	0.00 (± 0.00)	0.50 (± 0.58)
ETS	1.00 (± 1.41)	0.00 (± 0.00)	1.25 (± 1.26)
HDMA	3.60 (± 0.89)	0.20 (± 0.45)	1.60 (± 1.34)
ETS/HDMA	3.00 (± 1.16)	0.25 (± 0.50)	1.75 (± 1.50)

**Figure 2** Histology of supraglottic mucosal specimen in the control (FA) guinea pig treatment group. Note the relative lack of eosinophils (H&E, 200 $\times$).

Animals exposed to HDMA were 5.5 times more likely to have >10 eosinophils per high-power field in the supraglottis than animals not exposed to HDMA (95% CI, 1.57-19.27). Figures 2 and 3 contrast the marked eosinophils in the ETS/HDMA group compared with the control group.

Mucin

Table 5 displays the mean mucin indices stratified by exposure category. Exposure to HDMA and/or ETS did not appear to have any effect on the mucin index in the glottis or supraglottic larynx (one-way ANOVA *P* value = 0.34 and 0.60, respectively). Exposure to ETS and/or HDMA increased mucin production in the subglottis (one-way ANOVA = 0.01). The mean mucin index in the subglottis for the control group was 1.50 (± 1.73). This increased to 3.25 (± 0.96) in the ETS alone group, 2.80 (± 1.64) in the

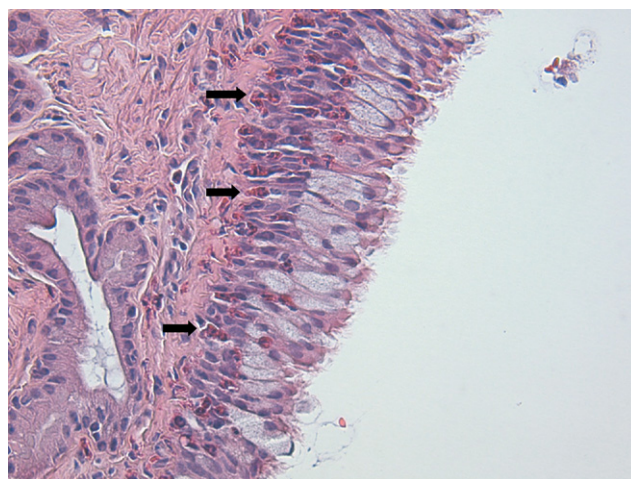
**Figure 3** Histology of supraglottic mucosal specimen in the ETS/HDMA guinea pig treatment group showing a marked increase (black arrows) in the number of eosinophils within the mucosa compared with the control group (H&E, 200 $\times$).

Table 5
Mean stored mucosubstance (mucin) index ($\pm$ SD)
by exposure group

Group	Supraglottis	Glottis	Subglottis
Control (FA)	1.25 ($\pm$ 1.26)	0.50 ($\pm$ 0.58)	1.50 ($\pm$ 1.73)
ETS	2.00 ($\pm$ 1.41)	0.00 ($\pm$ 0.00)	3.25 ($\pm$ 0.96)
HDMA	1.00 ($\pm$ 0.71)	0.00 ($\pm$ 0.00)	2.80 ($\pm$ 1.64)
ETS/HDMA	1.50 ($\pm$ 1.00)	0.00 ($\pm$ 0.00)	3.25 ($\pm$ 0.50)

HDMA alone group, and 3.25 ($\pm$ 0.50) in the ETS/HDMA group (MWU P value = 0.03, 0.11, and 0.03, respectively). Animals exposed to ETS and/or HDMA were 9.8 times more likely to have at least 25% of the subglottic epithelium stain for mucin (95% CI, 1.36–69.65). Figures 4 and 5 show the increase in the number of goblet cells in the ETS/HDMA group compared with the control group.

DISCUSSION

The larynx is exposed to a wide variety of environmental irritants including environmental tobacco smoke, laryngopharyngeal reflux, and pollutants. Despite the high frequency of laryngeal inflammation and pathology in the general population, the mechanisms whereby such challenges may affect the larynx have been largely neglected.

Our results establish that repeated challenges of an allergen using a standard protocol to induce allergic airway inflammation promote laryngeal eosinophilia in the supraglottic larynx of the guinea pig. This study also shows an association between HDMA and ETS exposure and elevated mucin in the subglottis. Previously published data have documented changes in laryngeal mucosa from various exposures and is in agreement with this work. Leonard et al^{5,6}

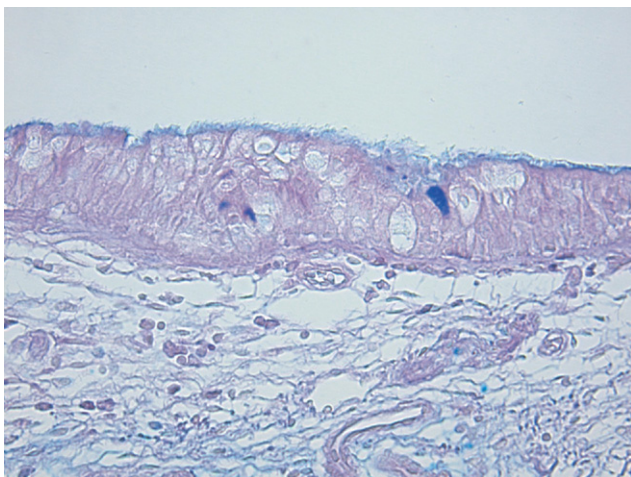


Figure 4 Histology of the subglottic mucosal specimen in the control (FA) guinea pig treatment group. Note the relative lack of stored mucosubstances (mucin) (Alcian-Blue 2.5, 200 $\times$).

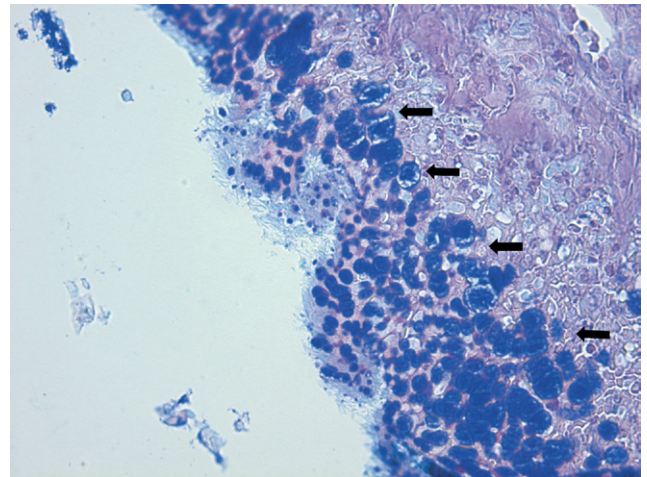


Figure 5 Histology of the subglottic mucosal specimen in the ETS/HDMA guinea pig treatment group. Images taken at 200 $\times$ show an increased number of goblet cells (black arrows) compared with the controlled as shown by Alcian blue 2.5 staining.

observed increases in the thickness of laryngeal epithelium, hyperplasia, and “disorganization” with exposure to ozone. These studies were not blinded and had an insufficient sample size to allow valid statistical comparisons to be made.

Naito et al⁷ evaluated laryngeal manifestations in Japanese cedar pollinosis patients. They showed that the severity of laryngeal symptoms varied directly with pollen counts, as did nasal and ophthalmologic symptoms. They proposed that cedar pollen might be a cause of allergic laryngitis in humans.⁷ To determine whether the allergic reaction of the larynx was actually caused by Japanese cedar pollen, Naito et al investigated rats immunized with one of the major allergens of Japanese cedar pollinosis. They found that rats sensitized and then exposed to cedar pollen induced laryngeal eosinophilia. The data were semiquantitative, and the counting performed was not blinded. Intranasal exposure to cedar pollen in sensitized rats was not increased by exposure to NO₂, although this exposure did increase serum levels of cedar-specific immunoglobulin E.⁷

Although the clinical significance of eosinophilia and elevated mucin in the larynx is not known, the data presented in this article provide further evidence to support the notion that environmental irritants contribute to the development of laryngitis. These data also support the notion that the larynx is an active participant in the unified airway. Histopathologic studies confirm that eosinophilia and mucin hypersecretion are characteristic findings of inflammation in both chronic rhinosinusitis and asthma.⁹ It is reasonable to hypothesize that these changes are also markers of laryngeal inflammatory disease.

Several limitations of this study should be noted. This was a relatively small study. Thus, although it has generated intriguing hypotheses, these require exploration in more detailed experiment with larger numbers. Power analysis suggests that to confirm that the effect of ETS/HDMA on

eosinophil score is real, eight animals per group would be required (two-tailed, 80% power, 5% level accepted). In support of the hypothesis raised that the finding of exposure to antigen increases laryngeal mucosal eosinophil numbers, however, studies of the lower airways have shown that combined exposure of ETS and allergens induces significantly increased levels of eosinophils compared with ETS or HDMA alone.¹⁰ In addition, further histopathologic studies of the larynx are necessary to evaluate the presence or absence of other key findings of inflammation such as major basic protein deposition, basement membrane thickening, and subepithelial edema. These quantitative studies have provided valuable insight on the mechanisms and relationships between ETS and allergens on the lower airways and would be quite useful to better understand the effects of these irritants on the larynx.¹⁰⁻¹²

It will be also interesting to study how the three subsites of the larynx (supraglottis, glottis, and subglottis) respond differently or similarly to the same irritants. In our study, the HDMA caused the greatest reaction at the level of the supraglottis with an increase in eosinophils, whereas ETS exposure appeared to influence mucin hypersecretion in the subglottis. The glottis that is composed of squamous epithelium and devoid of minor salivary glands was fairly resistant to both the HDMA and ETS exposures.

CONCLUSION

In this pilot study, exposure to house dust mite allergen was associated with eosinophilia in the supraglottic larynx. Exposure to house dust mite allergen and environmental tobacco smoke were associated with elevated mucin production in the subglottic larynx. These results provide further evidence that the larynx may be an active participant in the unified airway and that environmental tobacco smoke and inhaled allergens may be etiologic factors in the development of chronic laryngitis.

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AUTHOR CONTRIBUTIONS

Debbie A. Mouadeb, developed methods, conducted studies, reviewed results, wrote manuscript; **Peter C. Belafsky**, reviewed results, performed statistics, edited manuscript; **Martin Birchall**, developed methods, reviewed results, edited manuscript; **Carol Hood**, designed exposure and treatment protocol, oversaw experiments; **Thomas Konia**, reviewed pathology slides, captured figures; **Kent E. Pinkerton**, reviewed and oversaw exposure and treatment protocols, edited manuscript.

FINANCIAL DISCLOSURE

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