

ORIGINAL RESEARCH

Development of a murine model of chronic rhinosinusitis

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OBJECTIVE: The aim of this study was to develop a mouse model of chronic eosinophilic rhinosinusitis.

STUDY DESIGN: Mice were sensitized to *Aspergillus fumigatus* (Af) extract by intraperitoneal injection. The animals subsequently received nasal challenges with Af extract 3 times per week for 12 weeks. Sinonasal complexes were studied histologically by the study otolaryngologists and pathologists to characterize the inflammatory response.

SETTING: Animal care facility at an academic institution.

RESULTS: A chronic eosinophilic inflammatory response was evoked in all study animals. Statistical analysis was performed for inflammation, secretory cell hyperplasia, mast cells, and eosinophils. There were very significant differences ($P < 0.0005$) between control and study mice in all categories.

CONCLUSION: Prolonged nasal challenge of Af extract creates an inflammatory response in murine nasal mucosa that mimics human chronic eosinophilic rhinosinusitis.

SIGNIFICANCE: A murine model for chronic rhinosinusitis is reported that may facilitate future investigations into disease pathophysiology.

EBM rating: B-2

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Rhinosinusitis is one of the nation's most common and significant medical conditions. Although current medical and surgical therapies are effective in many patients,

they are not successful in all cases. A substantial number of patients continue to have persistent sinus symptoms that reduce quality of life. A deeper understanding of chronic rhinosinusitis pathophysiology is needed to improve treatment. Animal models have enhanced insight into many human diseases and also have been useful in testing new drug treatments. Currently, animal models for sinusitis are limited. In rabbits, a small surgical antrostomy, ostial obstruction, and bacterial inoculation are used to induce a bacterial sinusitis.¹ Mouse and rat models use inoculation and/or nasal foreign bodies to evoke a bacterial rhinitis/sinusitis.^{2,3} These models all cause an inflammatory infiltrate appropriate for bacterial infection, ie, neutrophils and lymphocytes. However, patients with chronic rhinosinusitis who do not respond to antibiotic or surgical therapy often have an inflammatory infiltrate characterized by mast cells, Th2 type lymphocytes, fibroblasts, and unregulated proliferation of eosinophils.⁴⁻⁶ In addition, histologic examination of their nasal mucosa shows damage to ciliated epithelium, basement membrane denudation, and secretory cell hyperplasia.^{4,6,7} These changes are similar to the chronic inflammatory changes that are well defined in the asthma literature.⁸ An animal model that reproduces this type inflammatory response within the nasal and/or sinus membranes is indicated. Recently, the established animal model for asthma has been adapted to study acute nasal diseases

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such as allergic rhinosinusitis.^{9,10} However, developing a chronic allergic animal model would be especially helpful in studying the cellular and genetic changes in chronic sinusitis. Developing this in a mouse would provide important advantages due to the vast number of species specific reagents available and the availability of genetically altered mice.

The primary purpose of our investigation was to establish a chronic inflammatory response within the nose/sinuses of a mouse, characterized by eosinophils and thereby establish a mouse disease model for chronic rhinosinusitis.

MATERIALS AND METHODS

Materials

A mixture of culture filtrate and mycelial extracts of *Aspergillus fumigatus* (*Af*) (Hollister-Stier Laboratories, Spokane, WA) was used for intraperitoneal and intranasal use.¹⁰ Seventy female BALB/c mice were obtained from Taconic Farms, Germantown, NY, and housed in pathogen-free conditions in the USUHS Laboratory of Animal Medicine until they were between 8 and 12 weeks of age. All procedures and animal care was conducted in accordance with federal regulations and policies.

Sensitization, Antigen Presentation

Thirty-five mice were controls and 35 were study mice. Study mice were subjected to an intraperitoneal injection of *Af* extract, 200 μ g absorbed into 2 mg of alum in .5 mL of phosphate buffered saline solution.¹¹ The other 35 mice acted as controls and received an intraperitoneal injection of 2 mg alum in .5 mL of phosphate buffered saline solution. A mixture of culture filtrate and mycelial extracts of *Af* was diluted to a concentration of 20 mg/cc with normal saline solution. One week after sensitization by intraperitoneal

injection, a nasal antigen challenge of *Af* extract was administered 3 times per week for 12 consecutive weeks to the study mice. The control animals received nasal saline solution at the same time intervals. Mice were lightly anesthetized by methoxyfluorane inhalation and 10 μ g (5 μ L) of *Af* antigen or 5 μ L of normal saline solution was applied to each nare by using a micropipette with the mouse held in the supine position. After instillation, the rodent was held upright until alert. Seven control and 7 study mice were euthanized at each study interval point of 0, 2, 4, 8, and 12 weeks for histologic analysis.

Histologic Analysis

Specimen preparation. The nose and sinuses were trimmed from the body and placed in formalin. After initial fixation for 24 hours in 10% neutral-buffered formalin, rodent skulls were skinned and incubated in decalcifying medium for 24 hours as previously described.¹⁰ After decalcification, cross-sections of the sinonasal structures were obtained. The skulls were sectioned coronally into 4 regions for paraffin embedding: (A) caudal to the incisor teeth, (B) halfway between A and C, (C) anterior margin of the orbit, and (D) posterior margin of the orbit.⁹ To characterize the inflammatory infiltrate, several stains were performed including hematoxylin and eosin, luna stain (for eosinophils), Giemsa stain (mast cells and eosinophils), Van Gieson stain and Masson's trichrome stain (for collagen), and mucicarmine and periodic acid-Schiff (goblet cells).

Anatomic method. Nasal diagrams¹² were used to standardize the anatomic locations being examined. First, the region with the vomeronasal organ was identified. The maxilloturbinate and the nasoturbinate were identified for anatomic orientation. Areas of olfactory mucosa were excluded from examination. Second, the region with the nasal-associated

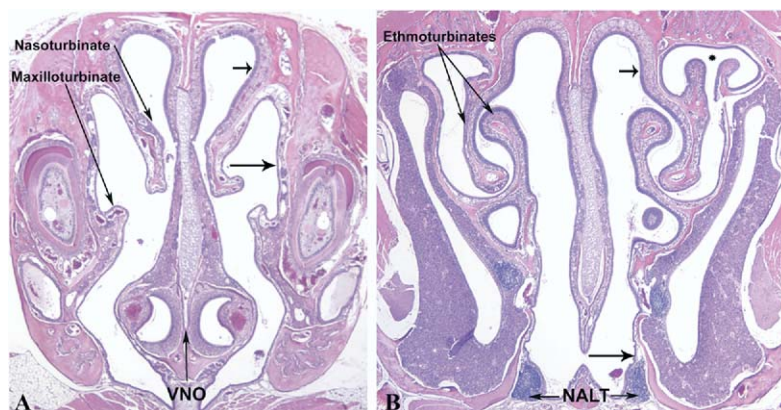


Figure 1 Reference nasal anatomy (hematoxylin and eosin stain; original magnification $\times 20$). (A) Vomeronasal organ section (VNO). Note the VNO, nasoturbinate, and the maxilloturbinate. The olfactory mucosa (*short arrow*) is located dorsally in the nasal cavity and on the dorsal septum while the respiratory mucosa (*long arrow*) is present on the lateral nasal walls and ventral septum. Squamous epithelium is present ventrally. (B) Nasal-associated lymphoid tissue section (NALT). Note the NALT and the highly branched ethmoturbinates. Mice do not have true sinus cavities but do have sinus-like cavities (*). The olfactory epithelium (*short arrow*) is located dorsally in the nasal cavity and on the septum; ventrally is respiratory epithelium (*long arrow*).

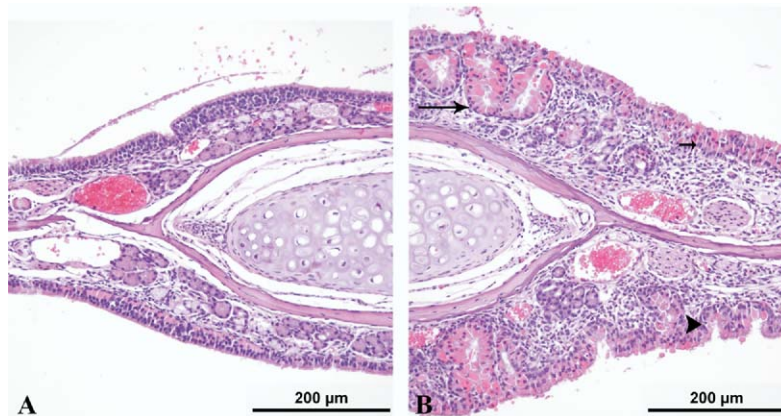


Figure 2 Inflammatory changes control vs study at low power (hematoxylin and eosin stain; original magnification $\times 200$). (A) (12-week control) Normal respiratory epithelium. (B) (12-week study) (1) Undulation of basement membrane zone (*arrowhead*), (2) size of lamina propria increased with inflammatory cells, (3) secretory metaplasia of septal glands (*long arrow*), and (4) hyperplasia of eosinophilic secretory cells (*short arrow*).

lymphoid tissue (NALT) was identified. The ethmoturbinates were identified and the regions of olfactory mucosa were excluded from examination (Fig 1). The 0 week slides served as histologic controls and showed ciliated respiratory epithelium, no eosinophils, and scattered mast cells (Fig 2A and Fig 3A).

Scoring. The presence and degree of inflammation was assayed by light microscopic examination. The control and study sinonasal cavities were examined with the examiner blinded to the experimental group and interval. All 4 histologic sections were reviewed. The best 2 sections, as determined by greatest sinus-like area included in the section, were scored for inflammation, secretory hyperplasia, eosinophils, and mast cells. Typically, this involved a section through the vomeronasal organ and a section through the NALT. Inflammation was evaluated by 2 criteria: the severity or grade of inflammation¹³ and the focal or diffuse nature of the inflammation. The severity or grade of inflammation was scored on a scale of none (0), minimal (1), mild (2), moderate (3), and severe (4). Minimal was defined as rare

individual inflammatory cells within the mucosa and submucosa, mild as a light infiltrate of individual and occasional clusters of inflammatory cells, moderate as a dense infiltrate of inflammatory cells, and severe as an inflammatory infiltrate so dense as to obscure the normal architecture of the mucosa or submucosa. The secretory hyperplasia was scored on a scale of none (0), minimal (1), mild (2), moderate (3), and severe (4). Minimal was defined as rare secretory cells, mild as scattered areas of secretory hyperplasia, moderate as diffuse areas of hyperplasia, and severe as diffuse areas of secretory cells so dense to obscure normal architecture and coupled with secretory metaplasia of subepithelial glands such as septal glands. Eosinophils and mast cells were scored on a scale of none/normal level (0), mild/slightly increased (1), and severe (2). The mean score from the 2 slides was determined and noted. The blinding code for the specimens was broken by the principal investigator and the inflammation scores and specimens to which they refer were organized by study group and time interval. For each time interval in each study group, the mean specimen inflammation scores were added to obtain a composite score. Com-

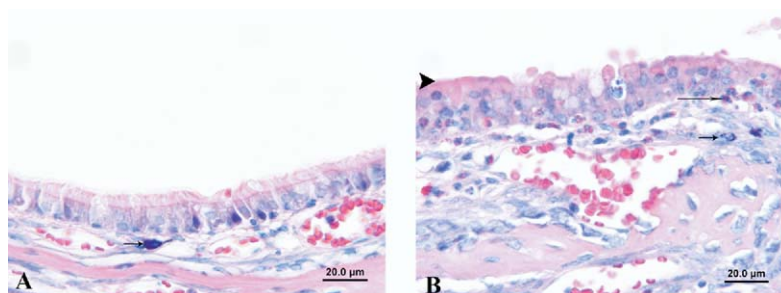


Figure 3 Inflammatory changes control vs study at high power (Giemsa stain; original magnification $\times 1000$). (A) (12-week control) (1) Normal respiratory ciliated epithelium, (2) absence of nonciliated epithelial secretory cells, (3) no eosinophilic infiltration, and (4) normal presence of one mast cell (*short arrow*). (B) (12-week study) (1) Loss of ciliated epithelium (*arrowhead*), (2) size of lamina propria increased with inflammatory cells, (3) nonciliated epithelial secretory cell hyperplasia with apocrine secretion, and (4) severe mast cell (*short arrow*) and eosinophilic (*long arrow*) infiltration with transmigration of eosinophils and globular leukocytes (epithelial mast cells).

posite scores at 0, 2, 4, 8, and 12 weeks were calculated and the control scores were compared with the study group scores and the study groups were compared with each other.

Statistical analysis. The composite scores were used to compare the control and study groups at all time intervals to determine if statistically significant differences exist. Two-way analyses of variance (2×5) were used to test for significant differences in inflammation, secretory cell, mast cell, and eosinophil scores. The first factor was group (study, control) and the second factor was weeks (0, 2, 4, 8, 12). A power analysis (Borenstein and Cohen, 1988) was computed to determine the power of various sample sizes for a 2×5 analysis of variance. With alpha equal to .05 and a large effect size (equivalent to a mean difference of .8 standard deviations between study and control groups), a sample size of 70 mice will give sufficient power (group main effect, power = .88; weeks main effect, power = .70; and group by weeks interaction, power = .70). One-way analyses of variance and Tamhane post hoc tests were performed on the experimental group to test for statistically significant differences between weeks for inflammation, secretory cells, mast cells, and eosinophils.

RESULTS

Very significant inflammatory and structural changes were seen between the control and study animals. Histologically, the study animals showed severe mast cell and eosinophilic infiltration with increased depth of lamina propria, loss of ciliated epithelium, hyperplasia of nonciliated eosinophilic secretory epithelial cells, and an undulating basement membrane zone (Figs 2B and 3B).

After 2 weeks of nasal antigen exposure, scattered eosinophils were noted in the lamina propria. Eosinophils were also noted within blood vessels, adhering to the internal luminal wall of blood vessels, and transmigrating through the blood vessel endothelial walls into the lamina propria. Epithelial cells showed apical cytoplasmic protuberances from the nonciliated cells (apical blebbing characteristic of apocrine secretion). Small (2 to 10 μm) eosinophilic globules are noted within the cytoplasm of the nonciliated epithelial cells. The overall stage of inflammation was mild to moderate and inflammation was largely multifocal. The level of secretory hyperplasia was mild and multifocal.

After 4 weeks of antigen exposure, there was an increase in transmigration of eosinophils through capillaries and an increase infiltration of the lamina propria by eosinophils. The nonciliated epithelial cells were increased as were apical cytoplasmic protuberances. Mast cells were seen within the lamina propria and variably staining globular leukocytes were seen within the epithelium.¹⁴ Small intracellular spaces appeared between epithelial cells. The overall stage of the inflammation was moderate, and inflammation was largely diffuse in nature. The level of secretory hyperplasia was moderate and diffuse.

At 8 weeks, the eosinophils, nonciliated epithelial cells, associated apical blebbing, and globular leukocytes continued to accumulate in the lamina propria. Mast cells were slightly more prominent within the lamina propria. Ciliated epithelial cells were decreased. The space between the epithelial cells also continued to increase. The overall architecture of the epithelium showed more disruption, the lamina propria was thickened, and the basement membrane zone was beginning to change from a linear to an undulating appearance. The overall stage of inflammation was moderate and inflammation was largely diffuse in nature. The level of secretory hyperplasia was moderate and diffuse.

After 12 weeks of antigen exposure, a severe diffuse chronic inflammatory condition was present. There was severe eosinophilic and mast cell infiltration into the lamina propria with transmigration of these cells through the epithelium. Lymphocytes were also increased. Globular leukocytes were also evident within the epithelium in significant numbers (Fig 4).¹⁴ The epithelium showed further signs of disruption with more frequent and pronounced spaces between cells, a thicker lamina propria, a more undulating basement membrane zone (Fig 5) and corresponding epithelial surface. However, true disruption of the epithelium and polyp formation were not evident. The epithelial layer was dominated by the nonciliated epithelial cells that showed a profound degree of blebbing (Fig 6). Crystals, similar in appearance to the Charcot-Leyden crystals found in human allergic fungal sinusitis, were present in regions of severe eosinophilic infiltration. Inflammatory exudate with eosinophilic predominance was seen in the nasal cavity and was associated with crystal formation (Fig 6). The level of inflammation was moderate to severe and diffuse in nature.

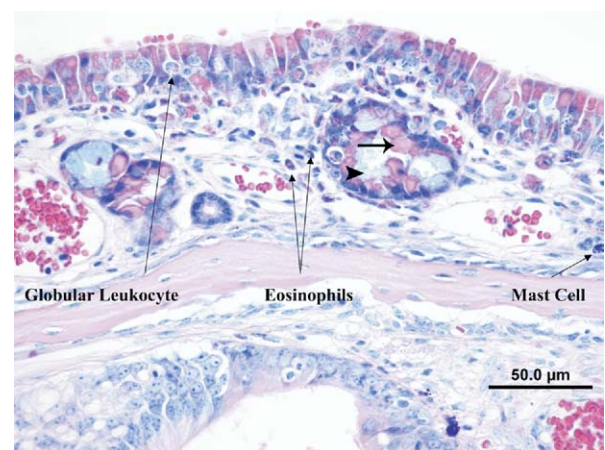


Figure 4 Cellular changes in 12-week study animal (Giemsa stain; original magnification $\times 600$, nasoturbinate). Note (1) severe level of inflammatory change, (2) hyperplasia of nonciliated secretory epithelial cells with apocrine secretion from these cells, (3) severe eosinophilic and mast cell infiltration, (4) transmigration of eosinophils and globular leukocytes (epithelial mast cells), (5) separation between epithelial cells, and (6) secretory cell metaplasia from mucous cells (arrowhead) to serous cells (long arrow).

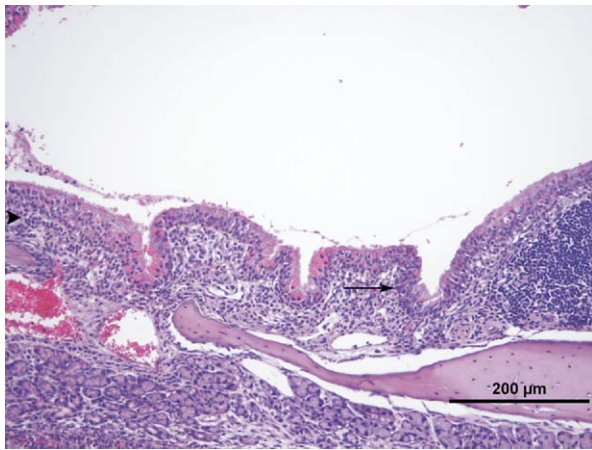


Figure 5 Possible pre-polypoid changes (12-week study animal, hematoxylin and eosin stain; original magnification $\times 200$, lateral nasal wall). Note the undulating basement membrane zone (long arrow) on the lateral nasal wall near the NALT associated with severe eosinophilic and mast cell infiltration in the lamina propria (arrowhead).

The level of secretory cells was severe with secretory metaplasia of subepithelial glands, especially septal glands.

Two-way analysis of variance was performed separately for inflammation, secretory cells, mast cells, and eosinophils. Each analysis showed very significant differences ($P < 0.0005$) in group, week, and week by group. Because there were no changes in the control groups, one-way analyses of variance and Tamhane post hoc tests were performed on the experimental group only to test for statistically significant differences between weeks for inflammation, secretory cells, mast cells, and eosinophils. Each one-way analysis of variance

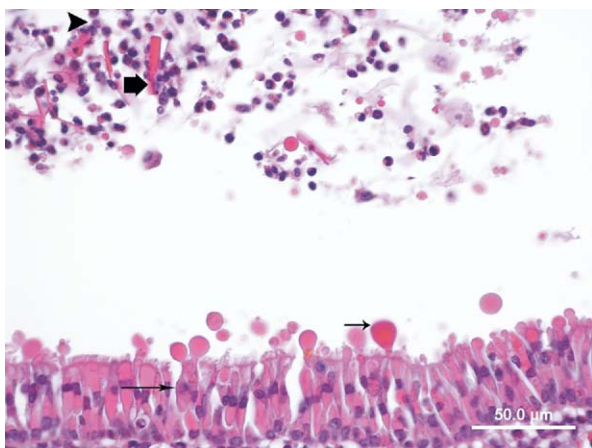


Figure 6 Apocrine secretion from hyperplastic nonciliated epithelial cells and inflammatory exudate with crystals (12-week study animal, hematoxylin and eosin stain, original magnification $\times 600$, area between the septum and the lateral nasal wall). Note the crystal formation (wide arrow) and the surrounding eosinophilic exudate (arrowhead). Also note the apocrine secretion (short arrow) from the nonciliated secretory epithelial cells (long arrow) and the hyperplasia of these cells.

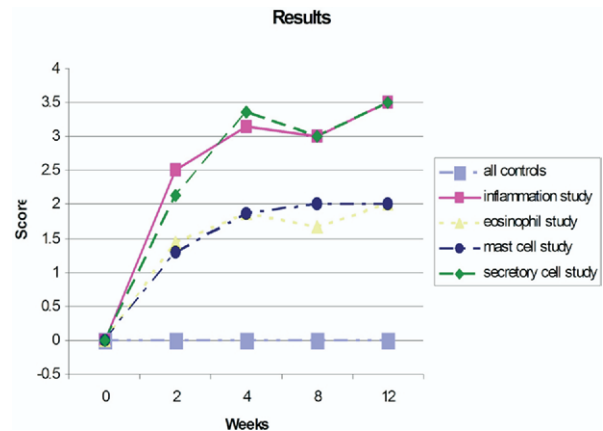


Figure 7 Estimated marginal means of inflammation, secretory cells, mast cells, and eosinophils. Two-way analysis of variance was performed separately for inflammation, secretory cells, mast cells, and eosinophils. Each analysis showed very significant differences ($P < 0.0005$) in group, week, and week by group. Because there were no changes in the control groups, one-way analyses of variance and Tamhane post hoc tests were performed on the experimental group only. Inflammation: Tamhane post hoc tests showed significant differences between week 0 and weeks 2, 4, 8, and 12. Secretory cells: Tamhane post hoc tests showed significant differences between week 0 and weeks 2, 4, 8, and 12, and between week 2 and weeks 8 and 12. Mast cells: Tamhane post hoc tests showed significant differences between week 0 and weeks 2 and 4. Eosinophils: Tamhane post hoc tests showed significant differences ($P < 0.0005$) between week 0 and weeks 2, 4, and 8.

showed significant differences between weeks for the experimental group ($P < 0.0005$). For inflammation, Tamhane post hoc tests showed significant differences between week 0 and weeks 2, 4, 8, and 12 (Fig 7). For secretory cells, Tamhane post hoc tests showed significant differences between week 0 and weeks 2, 4, 8, and 12, and between week 2 and weeks 8 and 12 (Fig 7). For mast cells, Tamhane post hoc tests showed significant differences between week 0 and weeks 2 and 4 (Fig 7). For eosinophils, Tamhane post hoc tests showed significant differences (< 0.0005) between week 0 and weeks 2, 4, and 8 (Fig 7).

DISCUSSION

We report the first successful model of chronic eosinophilic rhinosinusitis in the mouse. This model is based on the well-established lower airway acute asthma murine model and the recent murine model for acute allergic rhinitis.^{3,9-11} In our model, we present antigen to the sinonasal cavity for 12 consecutive weeks to meet the currently accepted clinical definition for chronic rhinosinusitis.¹⁵ Prolonged antigen presentation caused a significant inflammatory condition in the sinonasal cavities of study mice characterized by a significant eosinophilic submucosal infiltrate, an increased tissue mast cells/globular leukocytes, and a dramatic in-

crease in the number of nonciliated epithelial cells (secretory hyperplasia) that demonstrated marked apical blebbing (apocrine secretion), changes all found in chronic inflammation.^{4-6,8} Although the study specimens demonstrated many of the changes seen in human nasal polyps, including marked increase in eosinophils in the lamina propria, hyperplasia of secretory cells, disruption of intracellular attachments, and architectural changes in the basement membrane zone,⁷ no frank polyp formation was noted.

Murine models provide advantages for practicing scientists. Mice are inexpensive, easy to handle, and many species specific reagents are commercially available. Genetically defined mice are readily available offering a clear advantage over rat and rabbit models of sinusitis. Transgenic experimentation is providing startling advances in unlocking the basic pathophysiologic mechanisms of disease. Bringing this type of experimentation to rhinology could provide great insight into sinusitis and how to treat it better.

The selection of *Af* as the study antigen deserves special mention. The original airway model for inflammation used ovalbumin as the study antigen to produce asthma in mice. Recently, *Af* extract has been used in several murine models of upper and lower airway inflammation.^{10,11} *Af* has been implicated in the pathophysiology of chronic hyperplastic eosinophilic rhinosinusitis and in allergic fungal sinusitis.^{16,17} Therefore, as an established antigen in murine models and a possible factor in human disease, this antigen seemed likely to induce an experimental condition that could more closely mimic human sinonasal inflammation. In fact, we did find eosinophilic inflammatory exudate with crystal formation as is seen in patients with allergic fungal sinusitis.

The apical cytoplasmic protuberances (apical blebs) were a particularly interesting finding in this investigation. This finding has been previously described in sinonasal mucosa from human patients with perennial rhinitis¹⁸ and patients with chronic sinusitis.¹⁹ Given that this finding was experimentally induced, it suggests some degree of relevance of the experimental animal model to human disease. However, the extent and significance of this finding will need to be investigated further in additional studies.

Despite the advantages of the murine model, it has certain limitations. Mice do not have a sinonasal anatomic structure that is exactly comparable to humans, nor do mice have large sinus cavities that pneumatize into their skull and facial bones. They have very complex turbinate structures that increase the surface area of their olfactory epithelium.¹² More than half of a mouse's nasal epithelium is olfactory mucosa.²⁰ When studying the intranasal allergic response, investigators need to understand the normal distribution of respiratory epithelium and be able to distinguish inflamed respiratory mucosa from normal olfactory mucosa.¹² The histologic response in mice also includes a cell not typically described in the human allergic inflammatory response, the globular leukocyte.¹⁴ Histologic and electron microscopic studies show this to be a mast cell that leaves the submucosal tissue and transmigrates into the basal region of the epithelium.¹⁴

Investigators should avoid approaching murine research with the construct of human sinonasal anatomy. Although mice do not have true sinuses like a human this does not disqualify it as viable animal model in our opinion. Certainly when exploring the pathophysiologic mechanisms that pertain to ostial obstruction, a true sinus is needed and the rabbit sinus has served well. However, with more attention now on the immunologic response of sinonasal epithelium, a true sinus and experimental protocols that surgically obstruct this sinus are not as relevant.

Now that the model is established, additional investigations are needed to answer emerging questions. Can more prolonged antigen presentation or other modulating factors induce polyp formation? Further investigation to determine the time for spontaneous resolution of inflammation after termination of antigen presentation would also provide important information needed to establish a study baseline for testing new drug treatments. Eotaxin and IL-5 have been shown to be important in the influx of eosinophils into the nasal mucosa.^{9,10} Additional studies that complement and add to our histologic analysis, such as lymphocyte typing, cytokine and chemokine identification, and gene expression analysis can help ascertain more completely the nature of the immune response evoked in this model.

CONCLUSION

An animal model for chronic eosinophilic rhinosinusitis was successfully established by sensitizing mice to *Af* extract, and by presenting this antigen to their sinonasal mucosa for 12 weeks. Future investigations will help determine the model's ability to help investigators understand sinusitis and develop better treatments for patients.

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