

Allergic inflammation enhances bacterial sinusitis in mice

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Background: Although it is not proven, one factor considered important in the development of sinusitis is allergic rhinitis.

Objective: The purpose of this study was to determine whether ongoing allergic rhinitis enhances the infection and inflammation associated with *Streptococcus pneumoniae* acute sinus infection.

Methods: BALB/c mice were sensitized to ovalbumin by intraperitoneal injection. After infection of the sinuses by *S pneumoniae*, either with or without concomitant administration of ovalbumin to induce allergic inflammation, mice were killed at various times and their heads were prepared for histologic evaluation of the sinuses.

Results: Mice became allergic to ovalbumin and developed eosinophilia in the sinus and lung cavities in response to ovalbumin administration to each of the respective cavities. In comparison with controls, the mice with ongoing nasal allergic inflammation that were inoculated with *S pneumoniae* had significantly more bacteria recovered at sacrifice and had significantly more inflammation, as indicated by neutrophil, eosinophil, and mononuclear influx into the sinus mucosa. The percentage of the sinus area occupied by neutrophil clusters was also increased after infection in the allergic mice in comparison with the control mice.

Conclusion: Our data demonstrate that mice can be sensitized to ovalbumin and develop a localized allergic reaction in the skin, nose, or lung. An ongoing local allergic response augments bacterial infection in these animals. We also demonstrate that allergic sensitization alone, allergen exposure alone, or an allergic response at a distal site, the lung, does not augment the sinus infection. (*J Allergy Clin Immunol* 2001;108:424-9.)

Key words: Allergy, sinusitis, murine, ovalbumin, *Streptococcus pneumoniae*

Despite the significant morbidity associated with sinusitis and the enormous cost of treating it, little progress has been made in improving our understanding of the pathophysiologic nature of this disease. The main reasons for this paucity of progress are the limited ability to access the

Abbreviation used
OVA: Ovalbumin

sinuses of human subjects repetitively and the lack of a satisfactory animal model that mimics sinusitis in man.

Toward the goal of better understanding sinusitis, we have developed a mouse model of acute sinusitis after inoculation with *Streptococcus pneumoniae*, the most common pathogen responsible for acute sinusitis in man.^{1,2} We chose an unencapsulated serotype of *S pneumoniae* that causes infection and inflammation without causing mortality, in contrast to mouse models of peritonitis and pneumonia in which mortality is often the endpoint.³ Our goal was to mimic human disease in which otitis media and sinusitis, in contrast to invasive diseases such as pneumonia, are the result of noninvasive *S pneumoniae* disease.

Although it is not proven in human beings, one factor considered important in the development of sinusitis is allergic rhinitis. We use our mouse model to show that an ongoing allergic nasal reaction worsens acute bacterial sinusitis.

METHODS

Experimental animals

Pathogen-free 6- to 8-week-old BALB/c mice of either sex were purchased from Jackson Laboratories (Bar Harbor, Me). These animals were on an ovalbumin (OVA)-free diet and were kept in a pathogen-free environment. Each group of animals was kept isolated from the other groups in a biohazard containment facility. All animals were used in protocols approved by the Animal Care and Use Committee of the University of Chicago.

Allergen sensitization and exposure

OVA (Sigma Chemical, St Louis, Mo) for sensitization and exposure was prepared by dissolving 15 mg OVA in 75 mL of 0.1 mol/L PBS. OVA (1.5 mL) was then added to 1.5 mL of Alum Inject adjuvant solution (Pierce, Rockford, Ill). Sensitization of the animals was achieved by intraperitoneal injection of 200 μ L of the OVA solution in adjuvant (20 μ g OVA) on days 0 and 10 of the protocol. A localized allergic reaction was generated in the nose of each mouse by administration of a 6% OVA solution in 0.1 mol/L PBS on days 13 through 17 of the protocol. For the generation of an allergic reaction in the lungs, mice were administered the 6% OVA solution by a 15-minute aerosolization with a DeViliss nebulizer on days 13 through 17 of the protocol.

Intradermal skin test

The mice were skin-tested as previously described.⁴ Briefly, they were anesthetized with an intraperitoneal injection of ketamine/xylazine (80/8 mg/kg) 1 day before the skin test, and their

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undersides were shaved. We used tuberculin syringes to administer 50 μ L of 48/80 (a mast cell degranulating agent), BSA (an irrelevant antigen), or OVA (the sensitizing antigen), and wheals were measured 15 to 20 minutes after injection.

Blood collection

Blood was gathered from the orbital venous sinus after anesthesia was administered. The blood was spun at 5000 rpm, and the sera were collected and stored at -20° C until assayed.

Total IgE

Serum samples before and after allergy induction were assayed for total IgE through use of ELISA (Pharmingen, San Diego, Calif). Purified mouse IgE was added in 1:2 serial dilutions (100 ng/mL to 2000 ng/mL) to yield a standard curve. The plate was read at 490 nm, and values of the experimental samples were extrapolated off the standard curve, each value below 100 ng/mL being assigned a value of 50 ng/mL.

Inoculation

S pneumoniae (ATCC49619) was used for induction of acute sinusitis, as described previously.¹ The strain is antigenically similar to type 19 *S pneumoniae*, the most common strain cultured from human sinuses.³ The *S pneumoniae* was grown on blood agar plates, and colonies were suspended in sterile saline solution immediately before inoculation of the mice. A turbidity equivalent to McFarland no. 3 was used; this corresponds to approximately 1.2×10^9 colony-forming units per milliliter.¹ The mice were anesthetized with intraperitoneal injection of ketamine/xylazine, and 5 drops of the *S pneumoniae* suspension were placed in each naris. Experiments using inoculum varying by one log from 10^9 to 10^6 colony-forming units yielded similar bacterial counts (10^6) in lavage and neutrophil numbers on day 5. The mice were killed on day 5 after infection; prior experiments had shown peak infection in the sinuses at that time point.¹

In other experiments, cultures of homogenized lung and sinus tissues showed that the lungs consistently grew 100-fold fewer organisms. This finding is compatible with reports that nasal inoculation leads to pneumonia.⁵ Although this is true, the reason that our mice did not become ill and die was probably related to the serotype of the organism.

To address the concern that cultures obtained by nasal lavage did not reflect cultures from within the sinuses, we performed additional experiments. In 17 mice, we cultured the nose by lavage on day 5 after inoculation with *S pneumoniae* and then cultured the sinuses after sacrifice by removing sinus mucosa and homogenizing the tissue. The numbers of bacteria per gram of tissue ranged between 10^5 and 10^6 , levels compatible with those of an infection in human beings and with the findings on gram stains of the clusters (ie, the presence of organism on a gram stain implies at least 10^5 organisms).⁵ The correlation between the number of bacteria obtained by lavage and the number obtained from tissue cultures was robust and significant ($r = 0.85$, $P < 10^{-6}$). Because of the strong correlation, we feel comfortable using the lavage technique to quantify infection; we can then keep the sinuses intact for histologic evaluation of the degree of infection.

Allergy and infection study design

The mice were given intraperitoneal OVA/alum injections on days 0 and 8. They were then exposed to OVA intranasally or by aerosolization on days 9 through 13. All mice were inoculated intranasally with *S pneumoniae* on day 10. Mice were then killed on day 15. Control mice that were not sensitized with OVA were exposed to 1% PBS in the nose or lungs on the same days as the sensitized animals, infected intranasally with *S pneumoniae*, and killed 5 days after initiation of the infection.

Sacrifice and tissue harvesting and processing

On the day of sacrifice, the mice were sedated with a respiratory-failure dose of pentobarbital sodium (Nembutal) at 120 mg/kg. The final blood collection was obtained from the medial canthus, as described above, and the animal was transcardially (through the right atrium) perfused with lactated Ringer's solution; this was followed by perfusion with a solution of 4% formaldehyde and 0.5% glutaraldehyde in 0.1 mmol/L of phosphate buffer. When the experiments mandated it, the lungs were also harvested. Next, the animal was decapitated and the head prepared as previously described.¹ Sections of the nasal passages were cut at a thickness of 8 μ m, mounted on glass slides, and stained with Luna stain or hematoxylin and eosin.

Light microscopy and enumeration of inflammatory cells

Three anatomically similar sections were chosen from each mouse for analysis: an anterior section at the level of the maxillary sinuses, a middle section (more posterior and sampling the end of the maxillary sinuses and the beginning of the ethmoidal turbinates), and a third posterior section. Individual sections were analyzed after blinding by use of a computer-aided light microscope in conjunction with reconstruction software (NeuroLucida, MicroBrightfield, Vt). To quantify the degree of inflammation, we used 400 $\times$ magnification and traced the total sinus cavity area and the area of the sinus occupied by neutrophil clusters; this allowed us to calculate the percent of the sinus cavity filled with neutrophil clusters. Mucosa adjacent to neutrophil clusters was also traced and examined for polymorphonuclear cells, allowing us to report the number of cells per square millimeter. A random sampling of 4 mucosal areas from each of the 3 sections from each mouse was evaluated for the parameters described above, and the average of these measurements was computed for each mouse and used for statistical analysis. Eosinophils and mononuclear cells, as well as eosinophils in the lung, were counted in similar manner.

Nasal cultures

With the mice sedated, nasal lavage with 0.1 to 0.2 mL of sterile normal saline solution was performed. The lavage liquid was allowed to drip out directly from the nares onto Columbia blood agar and chocolate agar plates. For isolation, the nasal lavage sample was streaked perpendicularly 3 times across the plate. *S pneumoniae* growth on only the first streak represented few colonies; growth on the first and second streaks represented moderate colonies; and growth on the first, second, and third streaks represented many colonies.

Statistical analysis

We used parametric statistics to analyze the IgE and skin test data. ANOVA was performed; this was followed by a post hoc unpaired *t* test. For the other parameters, nonparametric statistics were used and a Kruskal-Wallis ANOVA was performed. Post hoc analysis was performed through use of the Mann-Whitney *U* test.

RESULTS

Sensitization to OVA

To ensure appropriate sensitization, we performed skin testing in sensitized and control mice (no sensitization and no challenge) and measured serum total IgE levels before and after sensitization in the actively sensitized group. The wheal elicited by skin testing showed reactivity to com-

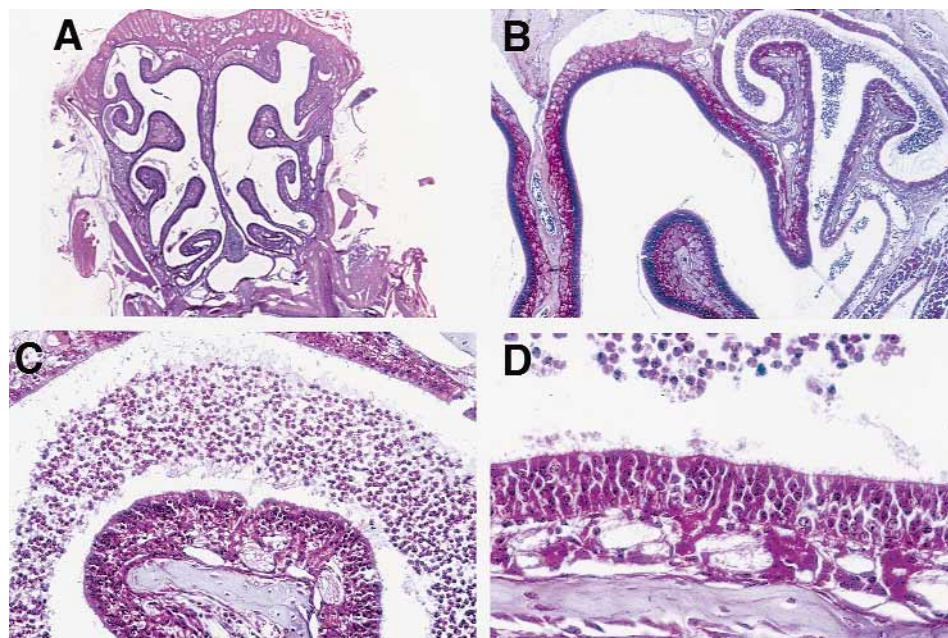


FIG 1. Representative examples of mouse sinuses stained with hematoxylin and eosin. **A**, 4 $\times$ magnification shows an entire coronal section through an infected mouse's sinuses. Superior is to the top of the figure. The midline structure is the septum. Turbinates and ethmoidal sinuses are also seen. Neutrophil clusters can be seen in some of the ethmoid sinuses. **B**, 16 $\times$ magnification illustrates a neutrophil cluster within an ethmoid sinus. Septum is seen to the left. Notice that the channel to the sinus is not obstructed. **C**, 128 $\times$ magnification shows the cluster in Fig 1, **B**. A gram stain of this cluster would show *S pneumoniae* organisms. **D**, 200 $\times$ magnification shows neutrophils within the mucosa around the cluster.

pound 48/80 in both control (4.6 ± 1.2 mm) and actively sensitized mice (5.2 ± 0.4 mm), no reactivity to the negative control (BSA) in either the controls (0.2 ± 0.2 mm) or the allergic (0.1 ± 0.1 mm) mice, and reactivity to OVA only in the sensitized mice, in which the increased was significant in comparison with the controls (5.2 ± 0.7 mm vs 0 ± 0 mm; $P < .01$, $n = 8$). There was a dramatic and significant increase in the serum levels of total IgE after sensitization with OVA (pre vs post: 0.8 ± 0.3 vs 42.5 ± 11.4 ; $P < .01$, $n = 8$); in contrast, the control group showed no significant change, most values being unmeasurable.

Induction of allergic inflammation

Next, we made mice allergic and exposed them to OVA administered by aerosol or intranasal inoculation for 5 consecutive days. The results are detailed in Table I. Results of ANOVA between the groups (A through I) for each of the parameters monitored were significant for sinus eosinophils ($P = .003$), lung eosinophils ($P = .0001$), sinus neutrophils ($P = .0001$), and the area of the sinuses occupied by neutrophil clusters ($P = .0002$). When post hoc analyses were performed, the most important differences were in the numbers of sinus eosinophils and sinus neutrophils, which were significantly higher in the mice that were actively sensitized and challenged with OVA intranasally (group E) than in any of the other groups (A, B, C, D, F, G, H, and I; $P < .01$ for eosinophils and neutrophils). The percent of the sinus area occupied

by neutrophil clusters was also larger in the mice sensitized with OVA and challenged with the same allergen than in most of the other groups (E vs A, B, C, G, H, and I; $P = .007$). Similarly, the number of eosinophils in the lungs was significantly higher in the group that was actively sensitized with OVA and challenged with OVA by the aerosolized route (H) than in any of the other groups (A, B, C, D, E, F, G, and I; $P \leq .02$).

Infection of allergic mice

We next asked whether an allergic reaction would augment the inflammatory response to *S pneumoniae*. We challenged 3 groups of BALB/c mice. The first group consisted of mice inoculated with *S pneumoniae* that were not made allergic. The second and third groups were sensitized, one group being challenged with antigen intranasally and the other group being challenged by aerosolization for 5 consecutive days. One day after the first antigen exposure, *S pneumoniae* was inoculated intranasally. All BALB/c infected mice demonstrated evidence of infection (Fig 1). The control/nonsensitized group and the sensitized group with pulmonary exposure showed similar degrees of intensity of inflammation, as assessed by the number of neutrophils per square millimeter of sinus mucosa and the percent of the sinus area occupied by neutrophil clusters. In contrast, the group of mice that were sensitized and exposed to allergen intranasally showed significantly increased parameters of

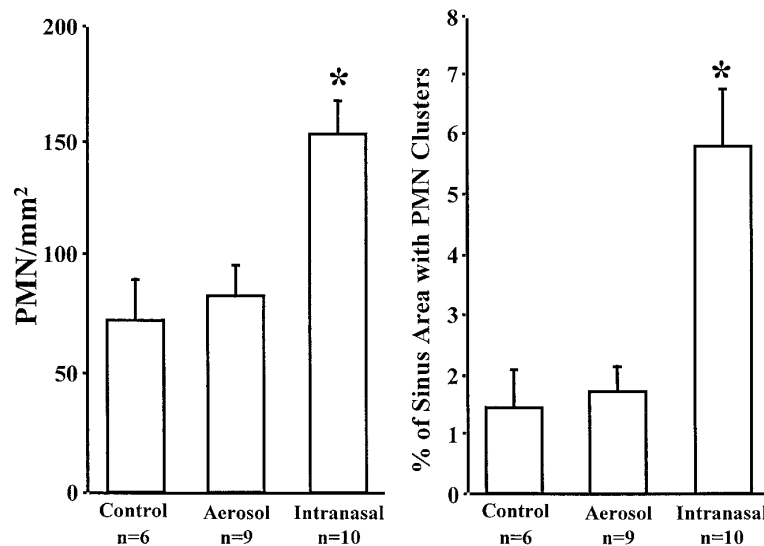


FIG 2. Inflammatory reaction in the sinuses after infection. The number of neutrophils/mm² of sinus mucosa (left panel) and the percent of the sinus surface area filled with neutrophil clusters (right panel) are shown in 3 groups of mice: those inoculated with *S pneumoniae* who were not made allergic (Control), those sensitized with OVA and infected simultaneously with OVA inhalation (Aerosol), and those sensitized with OVA and infected simultaneously with intranasal application of OVA (Intranasal). The results show that for both of the parameters assessed, the intensity of inflammation was augmented significantly in the group sensitized with OVA and infected in the presence of an ongoing nasal allergic reaction. * $P < .001$ vs Control and Aerosol.

inflammation, with an approximately 3-fold increase in the percentage of sinuses with neutrophil clusters and a 2-fold increase in the number of infiltrating neutrophils in the sinus mucosa ($P < .001$ vs controls and sensitized mice with lung exposure; Fig 2). As shown in Table II, a higher percent of lavages yielded moderate (50%) and heavy growth (30%) with intranasal allergen exposure in the sensitized mice than in the other 2 groups, suggesting a more severe infection in the former.

To control for a possible effect of the OVA other than in the induction of an allergic reaction, we repeated the experiment with one modification: this time the mice were not given intraperitoneal injections of OVA. The mice became infected and developed neutrophil clusters, but the results were not different from those seen in the control group and the clusters were fewer than those seen in the sensitized group given OVA intranasally.

Intranasal allergic mice with ongoing *S pneumoniae* infection showed a negative correlation between the eosinophil and neutrophil recruitment within their sinuses ($r = -0.62$; $P \leq .05$)—ie, the larger the number of eosinophils recruited by allergy, the smaller the number of neutrophils identified during acute infection. The total number of mononuclear cells in the sinus mucosa also increased significantly in infected mice in comparison with control mice ($P < .01$). These numbers were similar in allergic mice with ongoing infection and in sensitized mice made allergic with no infection. However, both the allergic/infected mice and the noninfected allergic mice had significantly higher numbers of mononuclear cells in the sinus mucosa than did either the controls or the mice with infection only ($P < .01$; Fig 3).

DISCUSSION

The exciting observation is that nasal instillation of *S pneumoniae* during ongoing nasal allergic inflammation augments the infection and the resultant inflammatory response. Allergic sensitization alone, allergen exposure, or an ongoing allergic reaction at another site did not augment the sinus infection, suggesting that it is the local inflammatory milieu that is responsible for this observation.

A number of factors in the human being are believed to be associated with an increased incidence of sinusitis, though none have been proved. These include allergic rhinitis, viral infections, cigarette smoke, anatomic defects, cystic fibrosis, air pollutants, antibody deficiencies, and ciliary dysmotility.⁶⁻¹³

In any large group of patients, allergic inflammation appears to be one factor that plays an important role in both acute and chronic sinusitis.^{8-10,14-17} Whereas there are no controlled evaluations comparing the prevalence of chronic sinusitis in allergic and nonallergic populations, there is a strong clinical impression suggesting an association between allergy and sinusitis. Van Dishoeck and Franssen¹⁸ estimated that allergy was an underlying factor in 40% to 67% of patients with chronic sinusitis. Studying outpatients in an otolaryngology clinic, Benninger¹⁹ found that 54% of those with chronic sinusitis also had allergic rhinitis. Grove and Farrior²⁰ found a 50% incidence of positive skin test results in patients undergoing sinus surgery, whereas Friedman²¹ reported an incidence of atopy in 94% of patients undergoing sphenoethmoidectomies. Savolainen⁹ found the incidence of allergy to be 25% in a group of 224 patients

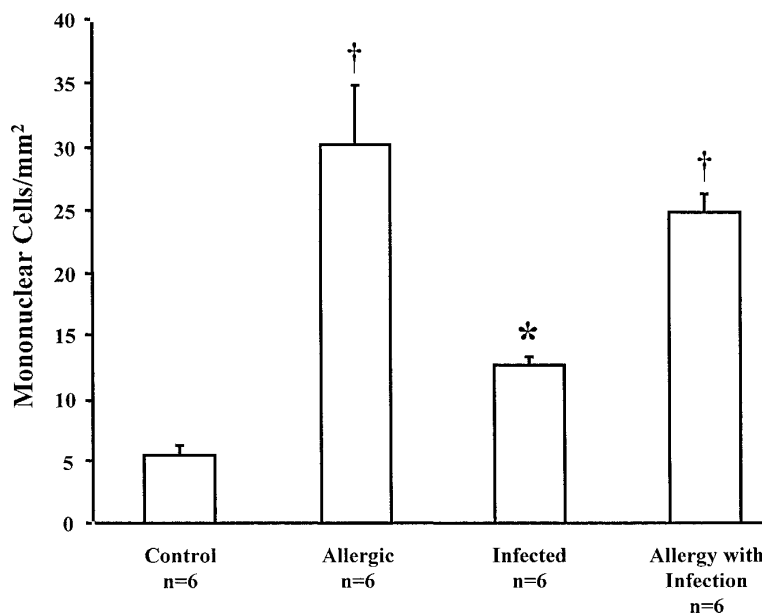


FIG 3. Numbers of mononuclear cells/mm² of sinus mucosa in control (noninfected), allergic, infected, and infected/allergic mice. There was a significant increase in the number of mononuclear cells in the infected mice in comparison with the controls (**P* < .01). The allergic mice had comparable numbers of mononuclear cells regardless of whether they were infected, and both groups had significantly more cells than did the control or infected-only groups (†*P* < .01).

TABLE I. Eosinophil and neutrophil responses of BALB/c mice to different antigen exposures

Group (no. of mice)	Sensitization	Inoculum	Sinus eosinophils (mean ± SEM)	Lung eosinophils (mean ± SEM)	Sinus PMNs (mean ± SEM)	PMN clusters/ percent sinus area
A (6)	Saline	Saline	12.4 ± 2.8	5.0 ± 1.4	10.5 ± 1.3	0 ± 0
B (6)	OVA/alum	Saline	12.0 ± 1.2	25.0 ± 6.5	10.1 ± 1.2	0 ± 0
C (6)	Alum	Saline	5.9 ± 0.6	9.7 ± 0.5	9.5 ± 1.9	0 ± 0
D (6)	Saline	Nasal OVA	14.5 ± 2.3	13.4 ± 1.6	16.6 ± 2.3	0.08 ± 0
E (6)	OVA/alum	Nasal OVA	380.3 ± 25	15.6 ± 3.3	66.9 ± 11.1	0.5 ± 0.002
F (6)	Alum	Nasal OVA	16.3 ± 1.4	24.4 ± 7.3	19.9 ± 3.1	0.06 ± 0
G (6)	Saline	Aerosol OVA	17.4 ± 1.5	45.5 ± 7.7	12.7 ± 1.2	0 ± 0
H (6)	OVA/alum	Aerosol OVA	20.0 ± 7.6	234.3 ± 70	16.4 ± 1.1	0 ± 0
I (6)	Alum	Aerosol OVA	18.7 ± 6.2	41.1 ± 3.6	11.7 ± 1.3	0 ± 0

TABLE II. Density of growth of *S pneumoniae* recovered by nasal lavage at sacrifice

	Density			
	None	Few	Moderate	Heavy
Control	0 (0)	4 (67)	1 (17)	1 (17)
Aerosol	1 (11)	6 (67)	2 (22)	0 (0)
Intranasal	0 (0)	2 (20)	5 (50)	3 (30)

Numbers of mice (%).

with acute maxillary sinusitis, which was significantly greater than the 16% incidence seen in a control group.

Complementing the data presented above, which indicate that allergic rhinitis occurs frequently in patients with sinusitis, are data showing a high prevalence of sinus disease in patients with allergic rhinitis. Sinus radiographs are abnormal in more than 50% of children who have perennial allergic rhinitis.^{14,15} In contrast, Enberg²² reported a low incidence of sinus abnormalities

in patients with perennial nonallergic rhinitis. MRI scans demonstrate increased evidence of sinus mucosal abnormalities during major pollinating seasons.²³ In human beings, acute sinus infections occur often during allergen exposure and are a common reason for the failure of allergy treatment.²⁴ In uncomplicated allergic rhinitis, other studies, using small numbers of subjects, found no imaging abnormalities of the sinuses.^{25,26}

How allergic rhinitis affects the course of sinusitis is not clear. It is conceivable, particularly in subjects with underlying anatomic defects, that allergen-induced nasal congestion could obstruct sinus drainage and enhance a subsequent bacterial infection. Anatomic obstructions within the sinuses were not present in our mice. Systemic differences between allergic individuals and nonallergic individuals might influence their responses to infection. We have observed that the responses to *S pneumoniae* in BALB/c and C57Bl/6 mice are not different.²⁷ Allergic inflammation, which increases the number of mucosal

mast cells, combined with C3a and C5a components generated secondary to complement activation in response to *S pneumoniae* infection, could lead to increased mast cell mediator release, causing greater transudation of fluid and increased proliferation of bacteria in the sinus cavities. Effects of inflammatory mediators released by eosinophils during an allergic reaction could alter the epithelium so that *S pneumoniae* binds better, leading to an augmented response. Ciliary transport from the sinuses could be altered by allergic inflammation, reducing clearance of bacteria and hence leading to an amplified infection. Allergic inflammation could "prime" circulating leukocytes in such a way that when there is recruitment of such primed cells to the sinus mucosa, an enhanced response to bacterial infection occurs. Allergic inflammation in the nasal cavity could induce neurogenic inflammation within the sinuses via central as well as axonal reflexes. Potential support for this concept comes from the study of Braunstahl et al,²⁸ in which antigen challenge of the lung caused an increase in the number of eosinophils in the nose. This observation seems to contradict our finding that aerosol challenge of the lung did not augment infection in the nose. The resolution of the contradiction might be the approximately 20-fold lower number of eosinophils at the distal site or the direct effect of another cell, mediator, or cytokine. A local mucosal response to infection involving T_H1 cells could be hindered by an ongoing local mucosal response to allergens involving T_H2 cells. Because of the ease of manipulation of the immune system in mice, our model should allow us better to investigate the relationship between allergic rhinitis and sinusitis.

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