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The Role of Ubiquitous Airborne Fungi in Chronic Rhinosinusitis

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Abstract

Chronic rhinosinusitis (CRS) is a confusing disease for both allergists and otorhinolaryngologists, partly because of its poorly understood pathophysiology and partly because of its limited treatment options. Several recent reports have provided evidence for a better understanding of the etiology and the relationship of CRS to airborne fungi—especially to *Alternaria*. First, the development of novel methods enables detection of certain fungi in mucus from the nasal and paranasal sinus cavities. Second, a non-IgE-mediated immunological mechanism for reactivity of patients with CRS to certain common fungi has been described. Third, these fungi are surrounded by eosinophils in vivo, suggesting that they are targeted by eosinophils. Finally, the preliminary results of studies using antifungal agents to treat patients with CRS are promising. Overall, these recent discoveries provide a logical mechanism for the pathophysiology of CRS, and they also suggest promising avenues for treatment of CRS with antifungal agents.

Index Entries

Chronic rhinosinusitis; sinusitis; fungi; molds; eosinophils.

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Introduction

A current report by the Centers for Disease Control and Prevention has estimated that the prevalence of sinusitis is up to 14.1% (29.2 million) of the US adult population, and the fact that there is no treatment for this disorder approved by the US Food and Drug Administration emphasizes the profound impact of this disease *(1,2)*. Patients with chronic rhinosinusitis (CRS) suffer from long-term nasal congestion, thick mucus production, loss of sense of smell, and intermittent acute exacerbations secondary to bacterial infections *(1,3)*. All of these symptoms can impair the patient's quality of life more severely than for patients with congestive heart failure *(4)*. In recent years, it has become clear that to understand CRS, one needs to examine the inflammation that exists within and outside the hyperplastic tissue.

Understanding the Inflammation

CRS is characterized histologically by an intense eosinophilic infiltration into the nasal mucosa *(5–7*,8)*. This eosinophilic inflammation occurs with or without nasal polyposis, is independent of atopy, and is neither present in healthy controls nor in patients with acute bacterial or viral sinus infections. The inflammation can be easily overlooked if patients are administered systemic steroids or other anti-inflammatory medication before harvesting the tissue for examination. This distinctive eosinophilic inflammation is also very heterogeneous (i.e., without eosinophilic infiltration in one area of a nasal mucosal tissue specimen, but with intense eosinophilic infiltration in another area of the same specimen) *(9)*. Therefore, reports in which only single biopsies are examined and in which it was unclear whether patients had received steroids before the biopsies were taken need to be interpreted carefully regarding the intensity of the eosinophilic infiltrate. Additionally, granule proteins from the eosinophils, such as major basic protein (MBP), that are toxic to sinus epithelium have been colocalized with the epithelial damage found in CRS *(7*,10)*. Recent in vivo observations have noted mostly intact eosinophils in the nasal tissues, but in the mucus, the eosinophils formed clusters, degranulated, and released their MBP at estimated levels far exceeding those needed to damage the epithelium *(11**)*. These in vivo observations explain the patterns of damage in CRS, where only the outer layers of tissue are damaged, suggesting that the damage to the epithelium is inflicted from the outside (luminal side). This epithelial damage may predispose patients with CRS to susceptibility for the secondary bacterial infections, leading to acute exacerbations, which are observed clinically. Because bacteria typically elicit a neutrophilic inflammation, these acute exacerbations of CRS are presumed to be of bacterial origin. However, the underlying eosinophilic inflammation that predominates in CRS is unlikely to be caused by bacterial infection, suggesting a nonbacterial etiological mechanism for CRS.

Eosinophilic inflammation has been observed in tissues that contain large, nonphagocytosable parasites (e.g., helminthes) *(12)*. Earlier reports have documented the accumulation of eosinophils and their subsequent degranulation on the surfaces of the parasites. The toxic proteins in the granule (including MBP) damage and kill the organisms. Recent observations of eosinophil clusters in mucus from patients with CRS have been reminiscent of the accumulations around parasites *(11**,13–15)*.

Two prospectively designed histological studies of mucus obtained during CRS surgery used extra caution to preserve the mucus. Eosinophilic mucus with clusters of aggregated eosinophils was found in 96% (97 of 101) and 94% (35 of 37) of consecutive patients with CRS *(14,15)*. Another study demonstrated that eosinophils released their toxic MBP in the mucus within these clusters and not in the tissue *(11**)*. Estimated concentrations of MBP within the clusters, based on digital analysis of the inten-

sity of the MBP staining, were as high as 2 m*M* and exceeded those capable of mediating epithelial damage. Overall, the clusters of eosinophils and intense eosinophil degranulation in the mucus suggest that eosinophils move from tissue to mucus with the fungi as their targets (Fig. 1).

A key issue questions whether the eosinophils in CRS play a defensive role similar to the role they play against parasites in their accumulation around extramucosal fungi. In other words, are eosinophils recruited to target fungi in the mucus of the patient with CRS? To answer this question, it is necessary to study whether certain fungi induce the recruitment, activation, survival, and degranulation of eosinophils from patients with CRS but not from healthy controls.

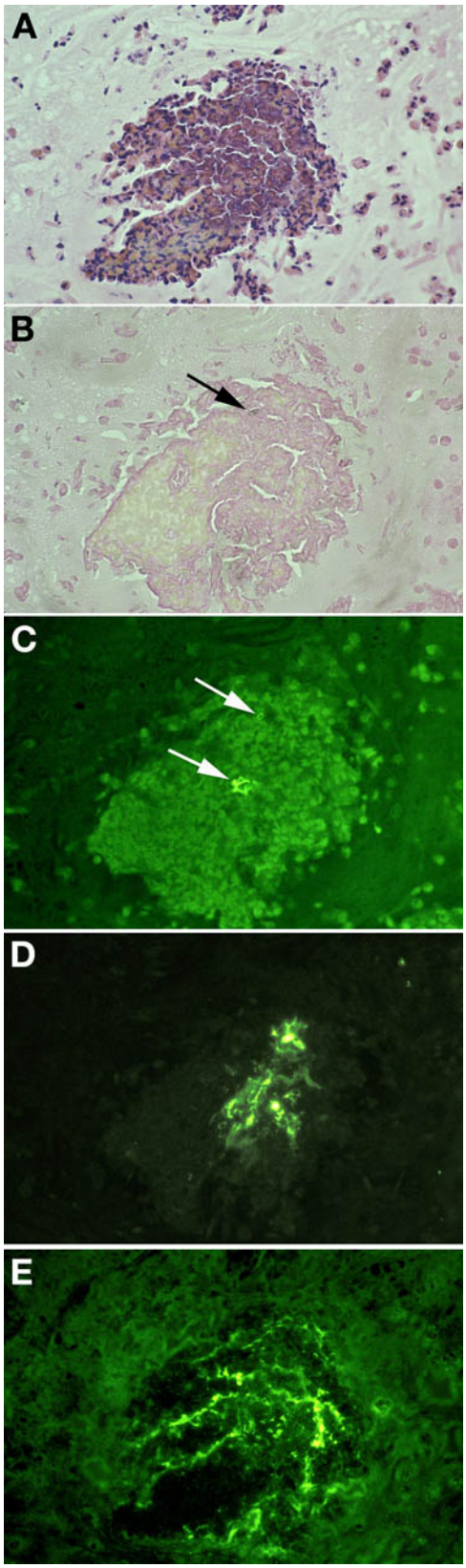

Fig. 1. **(A)** An example of an eosinophil cluster in the mucin of a patient with CRS, where eosinophils appear to target fungal organisms. This specimen was attached to sinus tissue and was removed during surgery. Because of the heterogeneous nature of the eosinophilia, where areas with and without eosinophils co-exist, these clusters can be easily missed on examination (hematoxylin and eosin counterstain of the same section shown in **E**; magnification ×800). **(B)** Serial section of the same cluster shown in **A**, stained with Gomori's methamine silver stain. The black arrow identifies a fungal organism (magnification ×800). **(C)** Serial section of the same cluster shown in **A** and **B**, stained for chitinase by immunofluorescence. Fungal elements (white arrows) are evident within the eosinophil cluster (magnification ×800). **(D)** Serial section of the same cluster shown in **A–C**, stained for *Alternaria* by immunofluorescence. Staining occurs both within and around fungal organisms (magnification ×800). **(E)** Same section as that shown in **A**, stained for eosinophil granule major basic protein (MBP) by immunofluorescence. Extracellular deposition of the toxic eosinophil granule protein MBP is evident within the eosinophil cluster (magnification ×800).

Where Are Fungi?

The role for fungi in CRS is noninvasive, and it is not a fungal infection. It needs to be differentiated from other forms of fungal sinusitis, such as fungus balls (noninvasive) and invasive fungal sinusitis (acute fulminant or chronic form). The fungi in patients with CRS are found only in the nasal mucus. Recently, striking progress has been made in the development of better techniques to detect fungi in the nasal secretions. To improve the sensitivity of fungal culture techniques, adequate amounts of mucus need to be harvested, and the disulfide bridges need to be chemically broken in the mucin to release the entrapped fungi *(14,15)*. Using these novel culturing techniques, newer studies have demonstrated the presence of fungi in 96% (n = 202) and 91.3% (n = 92) of mucus from unselected patients with CRS *(14,15)*. For the first time, these techniques have revealed the presence of fungi in almost every healthy control; this is not surprising because of the ubiquity of fungi in the air. Finally, fungi have been found in nasal secretions of healthy infants *(16)*. Immediately after birth, 20% of babies showed positive fungal cultures in their nasal secretions; at 5 d, 15% showed positive cultures; at 2 mo, 72% showed positive cultures; and at 4 mo, 94% showed positive cultures.

Increased awareness among pathologists and surgeons coupled with new techniques for mucus collection has resulted in higher detection rates of fungi on histology. An earlier comparison study showed a 47% failure rate in the demonstration of fungi and eosinophilic mucin in patients who were suspected for "allergic fungal sinusitis" *(17)*. More recently, with careful collection of nasal mucus specimens from patients with CRS and with Gomori's methamine silver stain, 82% (n = 101) and 75% (n = 82) of patients with CRS showed fungal elements in their mucus *(14,15)*. With the use of a chitin-based immunofluorescence staining technique, researchers found that 100% of nasal mucus specimens contained fungal elements *(18)*. Overall, the newer techniques for fungal detection have demonstrated increased specificity and sensitivity over old techniques to detect the presence of fungi.

Other techniques have been used by researchers to further enhance the detection of fungi. Polymerase chain reaction with specific fungal primers has been used to find fungal DNA in polypoid nasal tissues from 100% of patients with CRS (n = 27) *(19*)*. The authors speculated that this might represent fungal DNA that is being processed for antigen presentation. Interestingly, *Alternaria*-specific DNA was present in tissues from patients with CRS but not in tissues from healthy controls. Immunoassays detected levels of *Alternaria* antigens (ALT-a1) and total *Alternaria* proteins in the mucus of 100% of patients with CRS as well as in the mucus from all healthy controls *(20**,21**)*. These results are not surprising in view of a new study from the National Institute of Environmental Health Science, which has reported that virtually everyone is exposed to *Alternaria alternata* antigens at home *(22)*.

Because of newer collection, extraction, and detection techniques, fungi—specifically *Alternaria* species—are found in the nasal secretions of both patients with CRS and healthy controls. Therefore, the mere presence of fungi in the nasal and paranasal secretions does not appear to induce a chronic eosinophilic inflammation, nor is the presence of fungi alone diagnostic of a disease. However, to understand the presence of these organisms or their products, it is necessary for one to hypothesize that the inflammation in CRS might result from aberrant immune responses directed against certain fungi.

Why Do Eosinophils Exist? The Immune Response to Fungi

Although some patients with CRS produce specific IgE against fungi *(23,24)*, there is no

evidence that this IgE production results directly in the disease state of CRS. Furthermore, CRS develops in patients who both have or do not have IgE antibodies to fungi or other common aero-allergens. Conversely, IgE-mediated allergy and exposure to the relevant allergens leads to allergic rhinitis. Therefore, patients with CRS may have IgE-mediated hypersensitivity to molds as a comorbid disease, but the underlying eosinophilic inflammation appears to be driven by a mechanism independent from IgE mediation. If the human immune system in patients with CRS recognizes these fungi as foreign and uses eosinophils to attack them, then one could speculate that it should recruit and activate eosinophils by production of cytokines that regulate eosinophil inflammation. Tissue-bound lymphocytes have been found to be the main source of these cytokines in patients with CRS *(25)*.

Additionally, the expression of vascular cell adhesion molecule (VCAM)-1 has been identified in the vascular endothelium in patients with CRS *(26)*. This expression occurred independently of any IgE-mediated allergy and explains the presence of eosinophils in allergic as well as nonallergic patients with CRS *(26)*. VCAM-1 is known to specifically bind to the very late-appearing antigen-4 on eosinophils, thus causing selective adhesion and migration of eosinophils from the vasculature to the sinus tissue *(26)*. Shin et al. *(20**)* recently demonstrated that isolated peripheral blood mononuclear cells (PBMCs) from patients with CRS, which contained lymphocytes and other cells that can serve as antigen-presenting cells, produced large amounts of interleukin (IL)-13 when exposed in vitro to certain mold extracts—especially from *Alternaria* species. This production of IL-13 in response to *Alternaria* may enhance expression of VCAM-1 by vascular endothelial cells *(27)*.

Significantly elevated levels of IL-5, a cytokine that mediates eosinophil differentiation, survival, and activation, are present in tissue specimens of patients with CRS and not in those of healthy controls *(28–33**)*. A majority of the IL-5 staining cells are lymphocytes (68%), followed by eosinophils (18%) and mast cells (14%) *(34)*. PBMCs from 16 of 18 patients with CRS stimulated with *Alternaria* antigens in vitro showed increased production of IL-5, but PBMCs from 15 of 15 healthy controls did not *(20**)*. Elevated levels of specific IgE for *Alternaria* were detected in only 28% of these patients with CRS; the increased IgE levels did not correlate with increased levels of IL-5 *(20**)*. PBMCs from allergic and nonallergic patients with CRS produced similar amounts of IL-5, indicating that this reaction is independent from an IgE-mediated allergic reaction *(20**)*. Additionally, PBMCs from patients with CRS stimulated with either *Cladosporium* (6 of 18) or *Aspergillus* (4 of 18) antigens also showed increased production of IL-5; no response was seen with *Penicillium* antigen *(20**)*. Furthermore, production of interferon-γ, which facilitates destruction of parasites by eosinophils, is 5.5 times higher in PBMCs from patients with CRS stimulated with *Alternaria* antigen compared with production by healthy control PBMCs *(20**)*. These differences in cytokine production probably cannot be explained by differences in fungal contents in nasal mucus. When nasal secretions from nine healthy controls and nine patients with CRS were examined, there were no differences in their levels of total Alternaria proteins *(20**)*.

However, differences between patients with CRS and healthy controls exist in their immune responses to fungi. Mean serum IgG levels specific for *Alternaria* were increased about fivefold in the 18 patients with CRS compared with the 15 healthy controls *(20**)*. Furthermore, serum IgG levels in the patients with CRS directly correlated with the levels of IL-5 produced when patients' PBMCs were incubated with *Alternaria (20**)*. Because of the general notion that the levels of IgG indicate the amount of immunological exposure, these results

suggest a direct correlation between the exposure to *Alternaria* antigens and the severity of the immune reaction as determined by the amount of IL-5 production. Other investigators also found an increase of nasal obstruction after challenge with *Alternaria* that was independent from an IgE-mediated hypersensitivity *(35)*.

In a recent study, eosinophils from healthy people that were incubated with *Alternaria* and *Penicillium* antigens released significant amounts of eosinophil-derived neurotoxin, a marker of eosinophil degranulation *(36)*. When eosinophils from patients with asthma or allergies were used, they even released about 70% more eosinophil-derived neurotoxin compared with the healthy controls. The fraction from *A. alternata*, which induced the degranulation, had a molecular weight of approx 60 kDa, was highly heat labile, and worked protease dependant through a G protein-coupled receptor *(36)*. Other fungal antigens, including *Aspergillus*, *Cladosporium*, and *Candida*, did not induce eosinophil degranulation nor did neutrophils respond to *Alternaria* extracts, suggesting the presence of a fungal species and cell-type-specific novel innate immune response to certain fungi in human. Therefore, both innate and acquired immune responses to environmental fungi, such as *Alternaria* (independent of IgE antibodies to *Alternaria*) may increase production of the cytokines and provide cellular activation signals necessary for the robust eosinophilic inflammation in patients with CRS.

Antifungal Treatment of Chronic Rhinosinusitis

Because of the recent understanding of the association of numerous eosinophils with fungi in the mucus of patients with CRS, it now appears that antifungal agents applied directly to the nasal mucus could be beneficial. By reducing the fungal burden, the eosinophilia and accompanying inflammation might also be attenuated.

In one open-label pilot study, topical intranasal treatment of 51 patients with CRS, given as 20 mL of a 100-μg/mL amphotericin B solution per nostril, twice a day for average of 11.3 mo (range: 3–17 mo), improved both symptoms and endoscopic staging in 75% of patients and reduced mucosal thickening on available CT scans *(37)*. Ricchetti et al. *(38)* reported disappearance of polyposis on endoscopy in 62% of mild and 42% of moderate patients with CRS when topical amphotericin B was used for 4 wk. However, patients with severe CRS, who had polyps that filled the entire nasal cavity, showed no improvement *(38)*. In this severe CRS group, failure might have resulted from the limited access of the topical medication, or from the short duration of the therapy in the study, or both *(39)*. Similarly, a trial of patients with CRS who also had severe nasal polyposis used small volumes of amphotericin B and found no benefit after 8 wk *(40)*. When a bulb syringe was used as a delivery vehicle, a double-blinded, placebo-controlled trial of intranasal amphotericin B in an unselected CRS population found efficacy compared with placebo after 6 mo *(21**)*. Reduced inflammatory mucosal thickening could be demonstrated on CT scan and nasal endoscopy, and there were decreased levels of intranasal cytokines and markers for eosinophilic inflammation in patients with CRS. Recently, a group of patients with CRS was successfully treated with steroids and itraconazole in an open-label study, and no surgery was required *(41)*.

These results suggest that an antifungal treatment may reduce the fungal antigenic load in the nasal and paranasal cavities and, subsequently, may decrease the eosinophilic response. Overall, topical antifungal treatments likely benefit patients with CRS; this treatment needs to be long-term; and the dosage, formulation, and application methods still need further optimization, specifically in those with severely obstructive nasal and sinus passages.

A Broader Role for Fungi in CRS

New data have been presented that highlight the importance of certain airborne fungi—particularly the *Alternaria* species—in the pathophysiology of CRS. Although newer detection techniques have demonstrated the presence of these fungi in virtually everyone, only patients with CRS may react to them with the production of cytokines, which are crucial for the eosinophilic inflammation. Additionally, *Alternaria* extract induces a striking degranulation of eosinophils in vitro. Eosinophils apparently target fungi in vivo in the mucus of patients with CRS, where they degranulate within clusters. This extramucosal release of cytotoxic proteins may explain the damage observed to the epithelium, which is inflicted from the outside (luminal side), and the susceptibility of CRS to secondary bacterial infection (acute exacerbations of CRS). If antifungal therapy is used in the correct formulation, application form, and duration, it may be effective by inhibiting the antigenic load with a subsequent reduction of intranasal cytokines and markers of eosinophilic inflammation.

Acknowledgments

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