

Allergic fungal sinusitis: An immunohistologic analysis

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Background: Allergic fungal sinusitis is a noninvasive form of fungal sinusitis that has recently been delineated as a distinct clinicopathologic entity. It is increasingly recognized as a cause of chronic sinusitis, with the primary causative agents being members of the Dematiaceae fungus family. Although its immunopathogenesis has not been elucidated, the eosinophil is a prominent inflammatory cell on histologic examination.

Objective: We sought to characterize the involvement of eosinophils in sinus tissue and accompanying mucin from patients with allergic fungal sinusitis. As a comparison, neutrophil and mast cell involvement was also evaluated in the same group of patients.

Methods: Tissue specimens from 8 patients with allergic fungal sinusitis, along with 8 nasal polyp specimens from patients without allergic fungal sinusitis, were stained by using indirect immunofluorescence for eosinophil granule major basic protein (MBP). Neutrophil elastase and mast cell tryptase staining was also performed on the same allergic fungal sinusitis and nasal polyp tissues.

Results: MBP was diffusely localized within the mucin, showing intense staining at the periphery and variable staining of degenerated cell clusters throughout. Extracellular MBP in the mucin was strikingly greater than intact eosinophil staining. Diffuse extracellular neutrophil elastase was also present in the mucin. Mucinous areas showed no tryptase localization. Adjacent nonmucinous areas of respiratory mucosa showed predominantly cellular staining with eosinophil MBP, neutrophil elastase, and mast cell tryptase. MBP staining of nasal polyps showed a predominantly cellular pattern with focal areas of extracellular deposition.

Conclusions: Given the known toxicities of eosinophil granule MBP and neutrophil elastase, their extracellular presence supports the contribution of these proteins in the pathogenesis of allergic fungal sinusitis and further indicates that eosinophil and neutrophil activation occurs in the disease. (J Allergy Clin Immunol 2000;106:1096-101.)

Key words: Allergic fungal sinusitis, eosinophil, major basic protein, mast cell tryptase, neutrophil elastase

Fungal sinusitis may be classified as invasive or noninvasive.¹ Allergic fungal sinusitis is classified as nonin-

Abbreviations used

ECP: Eosinophil cationic protein
GMS: Grocott's methenamine silver
H&E: Hematoxylin and eosin
MBP: Major basic protein

sive disease and is the most commonly diagnosed and least understood form of fungal sinusitis. In 1981, Millar et al² first described 5 specimens from patients with chronic sinusitis that showed histologic similarities to allergic bronchopulmonary aspergillosis. Katzenstein et al³ independently reported 7 additional cases and named this entity allergic *Aspergillus* sinusitis. Since then, the majority of reports have shown non-*Aspergillus* fungi causing similar findings, prompting a change to the more general term, allergic fungal sinusitis. Fungal species reported in allergic fungal sinusitis include the following: *Curvularia*,^{4,5} *Alternaria*,⁵ *Exserohilum*,⁶ *Bipolaris*,⁷ *Chrysosporium*,⁸ *Rhizomucor*, and *Nodulisporium* species.⁹

Typical patients with allergic fungal sinusitis are immunocompetent atopic young adults with recurrent chronic sinusitis despite multiple surgical procedures. Nasal polyposis, asthma, elevated total IgE levels, and fungus-specific IgE and IgG levels, as well as peripheral blood eosinophilia, are characteristic of allergic fungal sinusitis.^{10,11} Diagnostic criteria have been put forth.^{12,13} Prevalence of the disease ranges from 4%¹⁴ to 7%^{7,15,16} of patients with chronic sinusitis. The diagnostic histologic feature is the presence of *allergic mucin*.¹⁷ Fungal hyphae are found within the allergic mucin and are best demonstrated with impregnated silver stains. Hematoxylin and eosin (H&E) staining of tissue specimens shows sloughed respiratory epithelial cells and necrotic cellular debris containing eosinophils and Charcot-Leyden crystals in the mucinous material. Polyps and pieces of inflamed respiratory mucosa are commonly found along with allergic mucin in sinus specimens.

The immunopathogenesis of allergic fungal sinusitis is not well understood. Type I hypersensitivity has been implicated in allergic fungal sinusitis on the basis of skin testing, fungus-specific RASTs, and total IgE elevations.^{13,18} However, a recent report challenges the importance of IgE-mediated reactivity in presumed allergic fungal sinusitis.¹⁹ Other groups have hypothesized that fungi become entrapped in the sinuses of individuals with ostiomeatal complex obstruction, extremely thick mucus, or a mucociliary clearance disorder, and the ensuing immune response to the antigen exacerbates the dis-

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ease in atopic patients.^{20,21} Eosinophils contribute to the pathogenesis of atopic diseases, such as atopic dermatitis^{22,23} and bronchial asthma,²⁴ and their cytotoxic granules potentially contribute to tissue damage, as well as disease severity, in allergic fungal sinusitis. Therefore we evaluated eosinophil presence and degranulation by using indirect immunofluorescence for eosinophil granule major basic protein (MBP) in sinus tissue specimens from patients with allergic fungal sinusitis. In addition, we compared MBP staining to neutrophil elastase and mast cell tryptase staining to further delineate the role of these inflammatory cells in this disease.

METHODS

Tissue specimens

After a review of patient histories and archived chronic sinusitis tissue sections at the Mayo Clinic,¹⁴ formaldehyde-fixed, paraffin-embedded tissue specimens from 8 patients with allergic fungal sinusitis were identified. These patients met the criteria for the diagnosis of allergic fungal sinusitis, including the presence of fungal hyphae in allergic mucin, as detected by Grocott's methenamine silver (GMS) stain, and positive sinus cultures, which grew various fungi, including *Alternaria*, *Aspergillus*, and *Curvularia* species. None of the 8 patients had evidence of invasive disease. Nasal polyps from an additional 8 patients were obtained during sinus surgery as control subjects. None of these nasal polyp tissues showed evidence of allergic mucin on histologic examination with H&E stains. Polyp specimens were fixed in 10% neutral-buffered formaldehyde and embedded in paraffin blocks. This investigation conformed to institutional review board regulations for conduct of human studies at Mayo Rochester.

Brightfield microscopy

Tissue sections (5 μ m) were cut from paraffin blocks of allergic fungal sinusitis and nasal polyp specimens, mounted onto glass slides, and stained with H&E and GMS.

Indirect immunofluorescence

Detection of eosinophil MBP, neutrophil elastase, and mast cell tryptase by means of indirect immunofluorescence was performed on formaldehyde-fixed, paraffin-embedded tissue sections, as previously described.^{22,23,25-27} Cellular MBP, elastase, and tryptase staining were scored on a visual analog scale of 0 to 3. A score of 0 indicated no immunofluorescent staining, a score of 1 indicated few intact cells staining, a score of 2 indicated several intact cells were present, and a score of 3 was assigned for infiltration of many cells. Extracellular MBP, elastase, and tryptase staining were also graded on a scale of 0 to 3. A score of 0 indicated no staining, a score of 1 indicated a few focal areas of staining, a score of 2 indicated focal areas of intense staining, and a score of 3 was given for extensive areas of intense staining.

Statistical analysis

Immunofluorescence staining grades were compared by using the Statview II (Abacus Concepts, Inc, Berkeley, Calif) software program with the Wilcoxon signed-rank, nonparametric, 2-group statistical test. Differences were considered significant for *P* values of less than .05.

RESULTS

Patient characteristics

Clinical characteristics of the 8 patients and 8 control subjects are shown in Table I. Patients ranged in age from 14

to 66 years (mean, 35 years). Half of the patients had asthma, and all but one had nasal polyposis. Five of the patients had previous sinus surgeries. Total eosinophil counts in peripheral blood were slightly elevated in 2 patients. Total serum IgE levels were elevated in 3 of 6 patients in whom IgE levels were measured. *Alternaria* species was the most common fungus isolated perioperatively on sinus culture. All of the patients from whom a fungus was identified on sinus culture had specific IgE to the isolated fungus determined by positive skin prick test responses or elevated allergen-specific IgE antibody test results.

Histology

All allergic fungal sinusitis tissues revealed copious amounts of pale eosinophilic mucin, clusters of degenerated cells, and laminated material (Fig 1, A). Two of 8 specimens consisted entirely of allergic mucin and amorphous tissue without identifiable intact respiratory mucosa, and the other 6 tissues had identifiable, edematous, respiratory mucosa with prominent eosinophil infiltration (Table II). GMS staining revealed scattered fungal elements in the allergic mucin of all specimens (Fig 1, B). No fungal elements were seen in the respiratory mucosa adjacent to allergic mucin.

Immunohistology

MBP, elastase, and tryptase were localized to cells in the respiratory mucosa adjacent to the allergic mucin (Fig 1). Extracellular MBP was found within the allergic mucin and was especially prominent at the periphery of mucin clusters, although scattered clusters of cellular debris were present throughout the mucin (Fig 1, C, and Table II). Within the allergic mucin, extracellular MBP deposition was striking, and few intact eosinophils were present, whereas in the respiratory mucosa intact eosinophils were relatively numerous, and extracellular MBP deposition was scant (*P* < .01). Mucin in all 8 specimens from patients with allergic fungal sinusitis had extracellular MBP present, whereas only 2 of the 8 specimens demonstrated intact eosinophils (Table II). Variable extracellular neutrophil elastase staining was also present in the allergic mucin and was disproportionately greater than the staining of intact cells (*P* < .02). Although extracellular MBP appeared more pronounced than elastase deposition in the mucin, this difference did not reach statistical significance (*P* = .088).

The allergic mucin showed no mast cell tryptase staining. Cellular tryptase was found only in adjacent respiratory mucosa and revealed a minimal-to-moderate number of mast cells. Extracellular tryptase in the respiratory mucosa was minimal (Fig 1, E).

Nasal polyps from patients with chronic sinusitis and without allergic fungal sinusitis showed moderate-to-many intact eosinophils by MBP staining (Table II). Extracellular MBP staining was also marked (Table II), ranging from focal areas of staining to areas of intense staining. Nasal polyp tissues are regularly used as positive control tissues for MBP assays in our laboratory, demonstrating both cellular and extracellular staining.

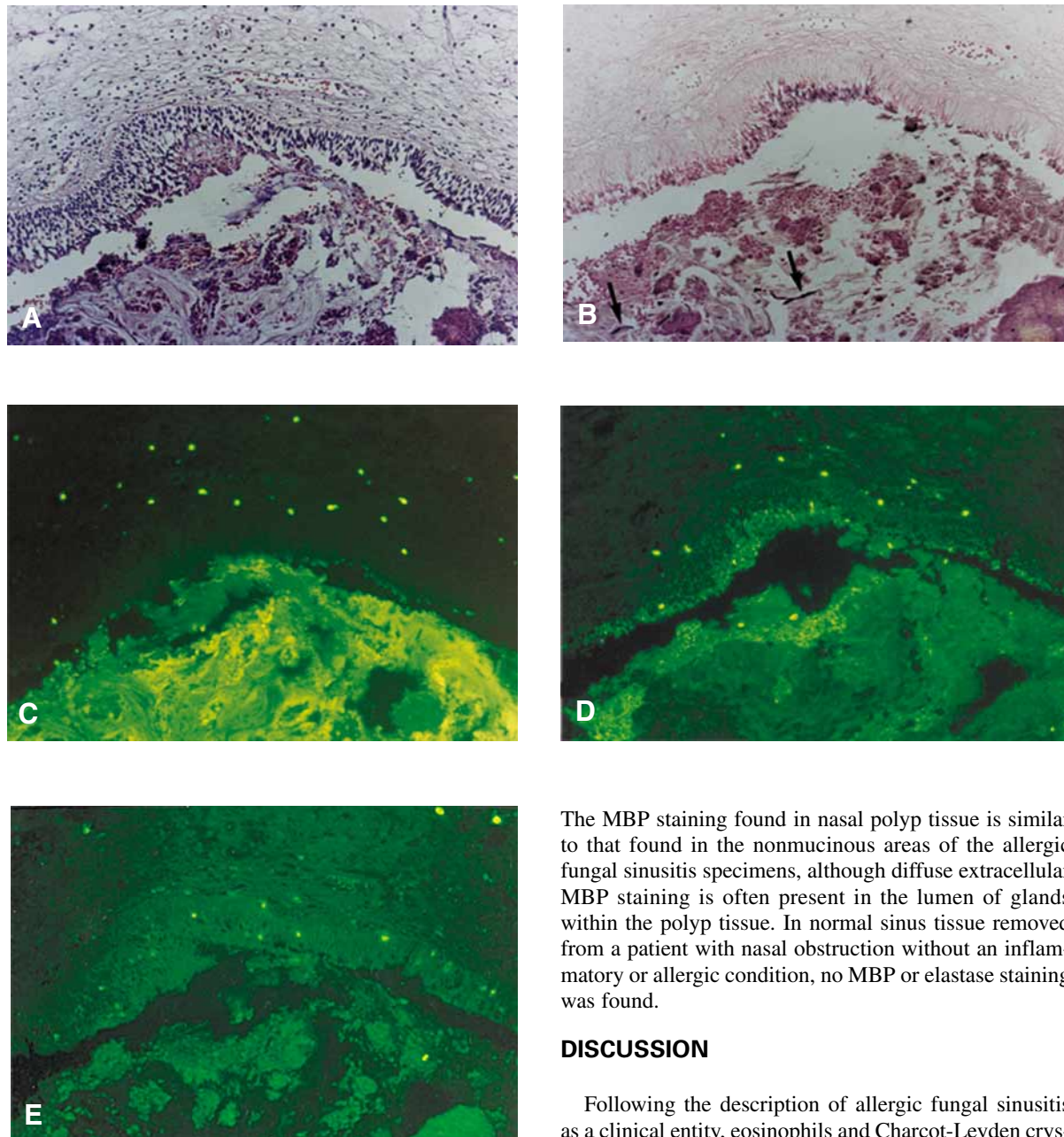


FIG 1. **A**, H&E staining of allergic fungal sinusitis tissue (patient 3), showing sinus mucosa (*upper*) and allergic mucin (*lower*) in an area in which the epithelium is mainly intact (original magnification, 160 $\times$). **B**, GMS staining of allergic fungal sinusitis tissue, showing fungal elements (*arrows*) within allergic mucin (original magnification, 160 $\times$). Sinus culture from surgery grew *Alternaria* species. **C**, Eosinophil granule MBP staining (patient 3, serial section of tissue in **A**), showing prominent extracellular MBP deposition in allergic mucin (*lower*) and predominantly cellular staining in sinus mucosa (*upper*; original magnification, 160 $\times$). **D**, Neutrophil elastase staining (patient 3, serial section of tissue in **C**), showing prominent extracellular deposition in allergic mucin and predominantly cellular staining in sinus mucosa (original magnification, 160 $\times$). **E**, Mast cell tryptase staining (patient 3, serial section of tissue in **D**), showing several mast cells in sinus mucosa without cellular or extracellular staining in allergic mucin (original magnification, 160 $\times$).

The MBP staining found in nasal polyp tissue is similar to that found in the nonmucinous areas of the allergic fungal sinusitis specimens, although diffuse extracellular MBP staining is often present in the lumen of glands within the polyp tissue. In normal sinus tissue removed from a patient with nasal obstruction without an inflammatory or allergic condition, no MBP or elastase staining was found.

DISCUSSION

Following the description of allergic fungal sinusitis as a clinical entity, eosinophils and Charcot-Leyden crystals have consistently been identified in specimens of allergic fungal sinusitis and, in fact, characterize the pathognomonic allergic mucin. The eosinophil granule contains several well-characterized cationic proteins that are potent cytotoxins.²⁸ In asthma and chronic sinusitis, MBP, the protein comprising the core of the eosinophil granule, has been found at sites of tissue destruction.^{24,29} MBP has been implicated in allergic inflammation by its ability to induce histamine release from mast cells and basophils³⁰ and to provoke a wheal-and-flare reaction when injected into human skin.^{31,32} The role of eosinophils in disease may not be adequately assessed by enumerating intact cells in tissue. Eosinophils often disrupt, depositing their toxic granule proteins, with loss of morphologic identity.²² Extracellular MBP has been

TABLE I. Patient and control characteristics

	Age (y)	Sex	Asthma	Polyps	Aspirin sensitivity	Prior sinus surgeries	Fungus species isolated	Blood eosinophils (cells/mm ³)*	Serum IgE IU/mL†
Patients									
1	66	F	–	+	–	0	+ (no ID)	280	43
2	32	F	–	+	–	2	<i>Alternaria</i>	ND	36
3	20	M	+	+	–	0	<i>Alternaria</i>	702	913
4	22	M	+	+	–	1	<i>Aspergillus</i>	470	993
5	26	F	+	+	–	2	<i>Alternaria</i>	270	ND
6	39	M	–	+	–	2	<i>Curvularia</i>	80	860
7	60	F	–	+	–	1	<i>Alternaria</i>	560	ND
8	14	M	–	–	–	0	<i>Alternaria</i>	194	316
Control subjects									
1	35	F	+	+	+	3	ND	ND	ND
2	28	F	+	+	+	8	Negative	1174	45
3	25	M	–	+	–	0	ND	ND	ND
4	55	M	+	+	+	0	ND	1020	63
5	37	F	+	+	+	8	ND	484	59
6	42	M	+	+	–	0	ND	1170	31
7	41	F	–	+	+	2	ND	ND	ND
8	53	M	–	+	–	0	ND	ND	ND

ID, Identification; ND, not determined.

*Normal level: 0 to 500 eosinophils/mm³.

†Normal level: 20 to 367 IU/mL.

TABLE II. Immunofluorescence staining

	1	2	3	4	5	6	7	8
AFS tissue								
Sinus respiratory mucosa								
MBP	1/2*	3/1	2/1	2/1	3/2	2/2	NA	NA
Elastase	0/2	2/0.5	2/1	0/1	1/0	2/1.5	NA	NA
Tryptase	2/0.5	1/0.5	2/0.5	0/0	1/0	2.5/0.5	NA	NA
Allergic mucin								
MBP	0/2	0/2	0/2	1/3	0/1.5	0/3	0/2.5	0.5/2.5
Elastase	0/2.5	0/1	0/2	0/1	0/1	0/2	0/3	0/1
Tryptase	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Nasal polyp tissue†								
MBP	3/3	2/3	1.5/1	3/3	2.5/3	2.5/3	3/2	3/2

AFS, Allergic fungal sinusitis; NA, not applicable (no nonmucinous tissue in specimen).

*Number grades are described in the "Methods" section (cellular grade/extracellular grade).

†Normal sinus tissue from a patient undergoing surgery for nasal obstruction was negative for MBP and elastase staining.

demonstrated in a number of diseases in which eosinophils likely have pathogenic effects, including asthma, chronic sinusitis, atopic dermatitis, chronic urticaria, and vernal conjunctivitis.^{22-25,27,29,33}

In this study of allergic fungal sinusitis, striking extracellular MBP staining was present in allergic mucin, particularly at the periphery, in the relative absence of intact eosinophils. This staining pattern differs from that in other types of sinusitis. Harlin et al²⁹ studied MBP staining in chronic sinusitis in patients with asthma, allergic rhinitis, or both and found prominent intracellular MBP staining that was also accompanied by extracellular MBP. Harlin et al also found a striking association between the presence of extracellular MBP deposition and damage to sinus mucosa. In another study Fujisawa et al²⁶ demonstrated marked intracellular and extracellular deposition of MBP in 10 sinus tissue specimens from patients with chronic sinusitis

and in 10 nasal polyp specimens, whereas neutrophil infiltration and extracellular elastase deposition were sparse.

The striking extracellular MBP staining in allergic mucin with few or no intact eosinophils suggests that eosinophils release their granule contents in the process of mucin generation. In contrast, mucosa/polyp tissues of these same specimens showed mainly intact eosinophils. Therefore the demonstration of striking extracellular MBP deposition in allergic mucin is not likely simply a reflection of concomitant eosinophil inflammation as found in nasal polyps or allergic rhinitis. A recent report by Feger et al³⁴ showed elevated levels of eosinophil cationic protein (ECP) in allergic mucin, providing additional evidence for eosinophil involvement. However, caution should be exercised in interpreting ECP levels as a specific marker for eosinophil involvement because ECP is also found in neutrophils.³⁵ In this study we

found evidence for neutrophil involvement with extracellular neutrophil elastase in allergic mucin. This may be associated with an inflation in the ECP levels because of a contribution from both eosinophils and neutrophils.

Elastase is a neutral serine protease with bactericidal properties present in the azurophilic or primary granules of neutrophils.³⁶ In addition to its beneficial effects, elastase has been implicated in tissue destruction associated with emphysema and other diseases.³⁷⁻³⁹ Here, extracellular elastase deposition was present in the allergic mucin of all tissues studied and tended to be less striking than MBP, although this did not reach statistical significance. Similar to the eosinophil findings, the allergic mucin areas did not show intact neutrophils. Adjacent polypoid nonmucinous tissue showed variable cellular elastase staining in 6 of 8 tissue specimens that contained nonmucinous tissue. The presence of extracellular elastase in the absence of intact neutrophils suggests that neutrophil degranulation occurs, potentially causing further damage to the tissues of these patients. To our knowledge, this is the first report of neutrophil products in allergic fungal sinusitis and suggests that neutrophils, in addition to eosinophils, are involved in the pathogenesis of this disease. Marked extracellular MBP and elastase deposition with limited intact cellular infiltration is similar to the pattern seen in the IgE-mediated, cutaneous, late-phase reaction.⁴⁰

Tryptase is a neutral serine protease present in the secretory granule of mast cells.⁴¹ Although the role of the mast cell in the early phase of allergic reactions is well established, its role in chronic allergic inflammation is not as well defined.⁴²⁻⁴⁴ IgE-mediated hypersensitivity has been proposed as contributing to the pathogenesis of allergic fungal sinusitis^{45,46}; therefore the mast cell, possessing a high-affinity IgE receptor, could be involved. Despite the presence of mast cells in surrounding sinus mucosa, no tryptase staining was detected in allergic mucin. Nonetheless, the possibility of mast cell involvement cannot be excluded on the basis of these results.

On the basis of histologic similarities to allergic bronchopulmonary aspergillosis and the immunohistologic similarities to the IgE-mediated, late-phase allergic reaction, one pathogenic theory for allergic fungal sinusitis is suggested. Ubiquitous fungal spores are inhaled and deposited in the upper airway mucosa of immunocompetent atopic individuals having asthma, nasal polyposis, or rhinitis with viscous secretions and thus are predisposed to trap fungal organisms. Certain patients manifest immediate hypersensitivity to the fungus,^{9,47} and IgE-fungal antibodies bound to high-affinity IgE receptors on mast cells are crosslinked by fungal antigens causing activation and degranulation. Subsequent release of various factors from mast cells and T cells, such as leukotriene B₄, leukotriene C₄, platelet-activating factor, and the cytokines TNF- α , IL-4, and IL-5 lead to tissue infiltration and activation of eosinophils and neutrophils.⁴² Toxic eosinophil and neutrophil granule proteins then contribute to tissue destruction and, ultimately, to the formation of allergic mucin.

The above theory is predicated on the presence of fungal-specific IgE. As mentioned above, the role of IgE in allergic fungal sinusitis has recently been challenged by Ponikau et al,¹⁹ who found no evidence of fungal-specific IgE antibodies in many patients to implicate IgE-mediated type I hypersensitivity. Because eosinophils and neutrophils can be recruited and activated and undergo degranulation through several IgE-independent mechanisms, eosinophil and neutrophil infiltration may still develop in the absence of IgE-dependent mast cell activation. The role of IgE-independent effector cell activation in allergic fungal sinusitis requires further investigation.

In summary, by using immunohistologic staining, this study provides additional information regarding the pathogenesis of allergic fungal sinusitis. The findings of prominent extracellular eosinophil MBP and, to a lesser extent, extracellular neutrophil elastase in allergic mucin strongly support a role for the eosinophil and the neutrophil in the pathogenesis of this disease. Although no tryptase staining was observed in allergic mucin, the possibility of mast cell involvement cannot be excluded on the basis of these studies. Further research on the mechanisms of eosinophil migration and degranulation in the allergic mucin along with eosinophil interactions with the invading fungi may help define improved treatments for this disease.

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