

Th2 immunological inflammation in allergic fungal sinusitis, nonallergic eosinophilic fungal sinusitis, and chronic rhinosinusitis

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ABSTRACT

Background: Noninvasive fungal sinusitis is a heterogeneous group of conditions including allergic fungal sinusitis (AFS) and nonallergic eosinophilic fungal sinusitis (NEFS). Th2-mediated cascades have been postulated to be the major inflammatory response in patients with AFS although other mechanisms also may be involved. The detailed mucosal Th2 cytological status of NEFS still has not been studied in great depth.

Methods: Using a meticulous patient selection algorithm over a 2-year period, infundibular mucosal tissue from patients with AFS, NEFS, chronic rhinosinusitis (CRS), and normal controls was studied ($n = 59$). Immunohistochemistry for mast cells, eosinophils, and immunoglobulin E (IgE) cells was performed and cell counts per unit area were measured.

Results: Mast cell, eosinophil, and IgE⁺ cell numbers were significantly raised in patients with AFS, NEFS, and CRS when compared with controls. There was no significant difference between cell numbers in patients with AFS and NEFS.

Conclusion: Patients with AFS exhibit a classic Th2 inflammatory response in nasal mucosal tissue with NEFS and CRS patients showing evidence of a similar Th2 cascade, including the presence of IgE⁺ cells. (Am J Rhinol 20:145–149, 2006)

Rhinosinusitis is an extremely prevalent condition and a significant cause of morbidity in patients.¹ As knowledge about the condition has advanced, the exact role of fungus in the pathophysiology of the condition has been thrust into the center of the debate.² In South Australia, 38% of patients undergoing sinus surgery have eosinophilic secretions suggestive of fungal disease with fungus being positively identified in 26%.³ This eosinophilic-rich mucin has the ability to promote the development of polyposis and the propensity to recurrence.⁴ Rhinosinusitis is a heterogeneous condition including various subtypes of noninvasive fungal rhinosinusitis. Where noninvasive fungus is present, allergic fungal sinusitis (AFS) and nonallergic eosinophilic fungal sinusitis (NEFS) constitute the two major groups of patients, the former with proven allergy to fungus, the latter without.³ AFS was first described in 1981 and the classic features of the condition are now well documented.⁵ Although AFS-like syndrome (with systemic immunoglobulin E [IgE] to fungus and rhinosinusitis but with negative culture) also has been described, it is a relatively rare condition in South Australia.³ The Mayo Clinic has shown that fungus can be cultured from the nasal cavity of the majority of patients with rhinosinusitis⁶ and this may be why AFS-like syndrome is now diagnosed less frequently than 10 years ago. As fungal stains and culture

techniques have improved⁷ and perhaps also owing to an increased clinical awareness of the importance of fungus in sinus disease, NEFS is now a frequently diagnosed condition³ although establishing the criteria required for a confident diagnosis of AFS, NEFS, or simply nonfungal chronic rhinosinusitis (CRS) is notoriously difficult.^{8–11}

As knowledge about the local synthesis of IgE in the nasal mucosa has increased,¹² now, it has been shown that many patients with nasal disease and negative skin-prick and/or normal systemic IgE will actually test positive on nasal challenges.¹³ In patients with idiopathic (nonallergic) rhinitis, specific IgE to allergens also can be localized within the nasal mucosa, despite no evidence of systemic atopy.¹⁴ The term “entopy” (Greek “*entopos*”—meaning local resident) has been proposed for the presence of local IgE in the absence of systemic disease.¹⁴ In patients with (nonatopic) NEFS, 19% have been shown to have antifungal IgE in the eosinophilic sinus mucin.¹⁵ This raises the possibility that, even in the absence of atopy, IgE⁺ cells still may be present in the nasal mucosa in patients with NEFS or even CRS. This study was designed to test this hypothesis.

METHODS

Before any tissue collection, ethical approval for this study was obtained from all appropriate hospital and university ethics committees. All patients gave their informed consent for tissue collection, which was obtained from January 2002 to December 2003.

Fungal Culture and Stain

Fungal staining was performed by using a combination of hematoxylin and eosin (H & E) and silver stains with cultures being performed by a single central laboratory using a modified three-plate technique that has been shown to optimize culture yields in our hands.^{3,16}

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Selection of Patient Groups

Controls. Control patients were patients undergoing endoscopic paranasal sinus surgery for endoscopic dacryocystorhinostomy who fulfill the following criteria:

1. Aged 18–65 years
2. Normal total IgE
3. Negative radioallergosorbent test (RAST) to standard aeroallergens
4. Negative skin-prick tests to aeroallergens
5. No asthma, eczema, or other chronic inflammatory disease
6. Normal macroscopic appearance of sinus mucosa at biopsy
7. No history of rhinosinusitis (British Society of Allergy and Clinical Immunology diagnostic history criteria)
8. No history of topical or systemic steroid or anti-histamine medication

AFS. AFS patients were patients undergoing endoscopic paranasal sinus surgery for CRS who fulfill the following criteria:

1. Aged 18–65 years
2. Raised total IgE
3. Positive RAST to fungus/mold mix or positive skin-prick test to fungi (≥ 1 of 6)
4. Polyps on rigid nasendoscopy
5. Computed tomography (CT) changes consistent with fungal sinusitis (polyps and mixed echogenicity of secretions)
6. Eosinophilic mucin without fungal invasion at time of surgery
7. Positive fungal culture and stain from sinus
8. No history of topical or systemic steroid or anti-histamine medication within 4 weeks

NEFS. NEFS patients were patients undergoing endoscopic paranasal sinus surgery for CRS who fulfill the following criteria:

1. Aged 18–65 years
2. Normal total IgE
3. Negative RAST/skin-prick to standard aeroallergens
4. Polyps on rigid nasendoscopy
5. CT changes consistent with fungal sinusitis
6. Eosinophilic mucin without fungal invasion at time of surgery
7. Positive fungal culture or fungal stain from the sinuses
8. No history of topical or systemic steroid or anti-histamine medication within 4 weeks

CRS. CRS patients were patients undergoing endoscopic paranasal sinus surgery for CRS who fulfill the following criteria:

1. Aged 18–65 years
2. Normal total IgE

3. Negative RAST/skin-prick tests to standard aeroallergens
4. No polyps on nasendoscopy
5. No CT changes consistent with fungal sinusitis
6. No evidence of eosinophilic mucin
7. Negative fungal culture or fungal stain from the sinuses
8. No history of topical or systemic steroid or anti-histamine medication within 4 weeks

Immunohistochemistry

Infundibular tissue from the face of the ethmoidal bulla or the uncinate process was sampled in all cases. Tissue was immediately snap frozen in isopentane (BDH, Poole, UK) suspended in liquid nitrogen and stored at -80°C until processing. Four-micron thick sections were used for immunohistochemistry. Endogenous peroxidases were blocked with 6% hydrogen peroxide for 2 hours at room temperature. Endogenous biotin was removed using an avidin/biotin blocking kit (Vector Labs, Peterborough, UK). Rabbit anti-human monoclonal antibodies against IgE (Dako, Glostrup, Denmark) and mouse anti-human mast cell tryptase (Dako) were visualized with streptavidin peroxidase, using diaminobenzidine as the chromogen (Chemicon, Temecula, CA). All sections were counterstained with Harris' hematoxylin.

Because of problems with the availability of monoclonal antibodies against major basic protein, eosinophils were localized with H & E and carbol chromotrope. The anti-human monoclonal anti-IgE antibody showed no cross-reactivity with IgA, IgG, IgM, or κ - or λ -light chain paraproteins.

Cell Counts

Sections were counted in a blind fashion by a single observer with a random sample recounted by a second individual with good reproducibility. Owing to a variable degree of damage to the epithelial layer because of biopsy techniques, cell counts were performed on the lamina propria immediately underlying the epithelium. Two sections and four random fields per section were analyzed for each patient.

Statistical Analysis

Mann-Whitney nonparametric analysis was performed on the mean cell counts (per high-powered field) for each patient group using GraphPad V4 (GraphPad, San Diego, CA). A probability of <0.05 was deemed significant.

RESULTS

Patient Numbers

Sixty tissue samples were analyzed in the study. Two blocks gave suboptimal and unreliable staining for eosinophils and IgE⁺ cells. Minimum patient numbers studied per group were AFS, 13 patients; NEFS, 18 patients; CRS, 17 patients; controls, 10 patients.

Eosinophils

Eosinophils were easily identifiable in the tissue of patients with sinus disease but rarely identifiable in controls. Although there was no statistical difference between the patients

with AFS and NEFS, there were significantly fewer eosinophils in CRS and controls (Table 1). Eosinophil counts are shown in Fig. 1.

Mast Cells

Mast cell counts (Fig. 2) were less variable than other cell counts between the groups. Patients with AFS and noninvasive fungal sinusitis had significantly more mast cells than controls. Mast cell counts also were increased in CRS patients but this increase did not reach statistical significance (Table 2). There were no significant differences between the three sinusitis patient groups.

IgE+ Cells

Although staining in lower numbers than eosinophils, a similar distribution pattern was seen between the groups (Fig. 3). All three patient groups showed significantly more cells than controls with noninvasive fungal sinus patients again having more cells than patients with CRS (Table 3). In one outlying control, individual significant numbers of IgE+ cells were visible.

DISCUSSION

In this study we tested the null hypothesis that there would be no difference in the Th2 inflammatory cell profile in the infundibular mucosa of patients with AFS, NEFS, CRS, and controls. Our results rejected the null hypothesis when symptomatic patients were compared with controls but failed to reject the hypothesis with a power of >90% when AFS patients were compared with those with NEFS.

Although patients with AFS have been studied extensively,^{5,17-19} there are fewer studies looking at patients with NEFS.^{3,4,15,20} It is more difficult to establish a “diagnosis of exclusion” for a detailed scientific study and detailed selection algorithms need to be constructed to avoid placing patients into the wrong clinical group.²¹ For this reason, it took 2 years to recruit patients for this study.

Chitin is found in the cell walls of fungi, and fluorescent staining to detect this protein is now established as an extremely sensitive method for detecting the presence of fungi within the paranasal sinuses.²² The antibodies required for this methodology unfortunately are not available within Australia and we were required to use a modified three-plate culture technique¹⁶ in conjunction with H & E and silver staining to detect fungi in this study. In our hands, this has optimized our ability to culture fungi on multiple plates in patients with eosinophilic mucin within the nasal sinuses although we accept its theoretical limitations. Therefore, it is

Table 1 Cell Counts and Statistical Analysis for Eosinophils

	Mean ± SEM	AFS	NEFS	CRS	Controls
AFS	20.1 ± 3.0	X	NS	0.003	<0.0001
NEFS	14.3 ± 2.4	X	X	NS	<0.0001
CRS	8.1 ± 2.1	X	X	X	0.001
Controls	0.2 ± 0.2	X	X	X	X

NS = not significant.

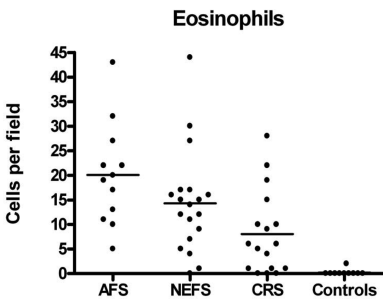


Figure 1. Scattergram showing the distribution and mean eosinophil cell counts. See Table 1 for statistical analysis.

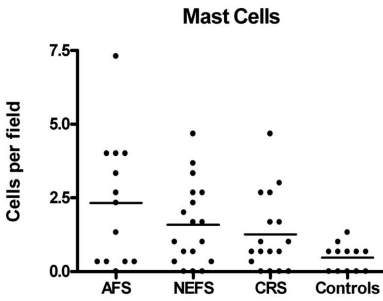


Figure 2. Scattergram showing the distribution and mean mast cell counts. See Table 2 for statistical analysis.

Table 2 Cell Counts and Statistical Analysis for Mast Cells

	Mean ± SEM	AFS	NEFS	CRS	Controls
AFS	2.33 ± 0.60	X	NS	NS	0.03
NEFS	1.59 ± 0.33	X	X	NS	0.02
CRS	1.23 ± 0.32	X	X	X	NS
Controls	0.47 ± 0.13	X	X	X	X

NS = not significant.

theoretically possible to criticize our methodology by claiming that some patients with AFS or NEFS might have been categorized incorrectly into the CRS group. However, the lack of eosinophilic mucin was included in the inclusion criteria for the CFS group and therefore it is more likely that patients with false negative fungal detection were simply inappropriately excluded from the study.

Although sinus irrigation, cytological brushings, or other cell-sampling techniques²³ could have been used for this study, we chose to obtain full-thickness mucosal biopsy specimens because the cellular interactions within the submucosa are pivotal in understanding the pathophysiology of any nasal inflammatory response.²⁴ These mucosal biopsy specimens were taken from the heart of the osteomeatal complex, so as to obtain tissue from the area where maximal inflammation was most likely to be apparent.

Eosinophils

Eosinophils are thought to be the key cellular marker for fungal sinusitis.¹⁹ They are produced in the bone marrow,

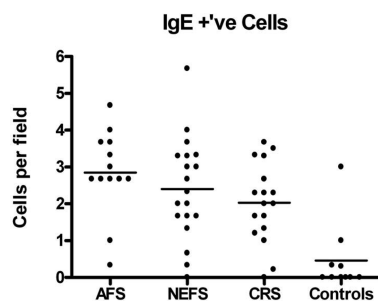


Figure 3. Scattergram showing the distribution and mean IgE⁺ cell counts. See Table 3 for statistical analysis.

Table 3 Cell Counts and Statistical Analysis for IgE⁺ Cells

	Mean ± SEM	AFS	NEFS	CRS	Controls
AFS	2.8 ± 0.32	X	NS	0.02	<0.001
NEFS	2.4 ± 0.32	X	X	NS	0.002
CRS	2.0 ± 0.26	X	X	X	0.002
Controls	0.46 ± 0.30	X	X	X	X

NS = not significant.

migrate into the vascular system, and are trapped in the vessels of the nasal mucosa by adhesion molecules. This whole process is controlled by Th2 procytokines, most importantly interleukin-5.^{25,26} Patients with fungal balls (mycetomas) do not show the same excessive eosinophil response despite there being a major fungal load within the sinuses.²⁷ It also is known that patients with raised serum eosinophilia in nonfungal CRS have a worse prognostic outcome.²⁸ Eosinophil secretion in response to parasite infestation is a normal physiological response and yet there are obviously host factors that determine the level of this response. If a small fungal load is successfully killed off by eosinophils and thick mucous, then no fungus will be cultured from the sinus and staining also may fail to identify the original cause of the eosinophilic mucin production.⁷ Therefore, it is possible that the eosinophilic response seen in patients with CRS was caused initially by fungus as also has been proposed by other authors.^{2,6,9} In addition, eosinophil numbers are increased in the late-phase allergic response²⁹ and eosinophilic sinus mucin has been simplistically termed “allergic” mucin⁸ for many years. In a detailed study looking at patients with perennial allergic rhinitis and idiopathic (nonallergic) rhinitis, no significant difference was seen in submucosal eosinophil numbers when full-thickness mucosal samples were studied.²⁹ Although AFS always has been presumed to be driven by an “allergic” inflammatory cascade,³⁰ there is actually little evidence to suggest that the atopy to fungus is anything more than an amplification factor.^{2,31} Our data suggest that AFS and NEFS (and to a lesser degree, CRS) have similar eosinophilic responses that do not rule out the possibility of local IgE production as a local amplification factor for the inflammatory cascade.

Mast Cells

Mast cell numbers are increased in allergic rhinitis and are an important part of Th2-mediated inflammation.³² They are a major source of interleukin-4, which drives IgE production,¹² and mast cell numbers are known to be markedly reduced by topical and systemic steroids. In this study, the differences between groups was less marked with respect to mast cell numbers than to the other types of cells studied. Our data show that noninvasive fungal sinusitis does produce increased numbers of mucosal mast cells and that these cells, which are known to have a pivotal role in increasing IgE production in local B cells,¹² may be responsible for IgE regulation at a mucosal level in both AFS and NEFS. When looking at patients with rhinitis, mast cells do seem to have close cellular interactions with T-cell subsets (especially CD8⁺) within the nasal mucosa. These interactions are present irrespective of the atopic state of the patient with rhinitis, implying that regulation of the T cells is not systematically controlled but is more likely caused by local factors within the mucosa.²⁴ Steroid therapy is known to be important for giving continued symptom relief after surgery for fungal sinusitis.⁵ The findings of raised mast cell numbers in these untreated patient groups correlates with the clinical behavior of the conditions and their response to steroid therapy.

IgE⁺ Cells

IgE⁺ cells can be mast cells or plasma cells and in patients with systemic allergy, it is well known that nasal mucosal plasma cell numbers are significantly raised.²⁹ Although it is well known that IgE is raised in AFS, levels are not raised in the systemic circulation of patients with NEFS or (with the selection criteria for this article) CRS and but all patient groups in this study showed significantly more IgE⁺ cells than controls.

There is an increasing body of evidence that the absence of atopy does not mean that IgE is not involved in inflammation at a mucosal level within the nasal cavity. Selective B-cell switching occurs within the nasal mucosa,¹² patients with nonallergic rhinitis test positive on allergen challenge¹³ and show autoantibodies to IgE,³³ specific IgE to grasses can be found in the nasal mucosa of patients who are serologically and skin-prick test negative,¹⁴ and patients with non-AFS show antifungal IgE in sinus secretions.¹⁵ Our whole concept of atopy, allergy, and now “entropy” is constantly evolving as we discover more about the local cellular interactions that occur within the nasal mucosa. Noninvasive fungal colonization of the sinuses adds an additional complexity to the cytological changes that occur and more work needs to be done to look more closely at cytokine production and targeting, intracellular interactions, and *in vivo* mucosal responses in these patient groups.

CONCLUSIONS

There has been little work looking at full-thickness mucosal tissue in patients with the various subtypes of rhinosinusitis. Our findings have shown that patients with AFS, NEFS, and CRS all show increases in Th2 cellular markers compared with controls. The raised numbers of IgE⁺ cells in NEFS and CRS adds further evidence to the possibility that local IgE

production or “entropy” may be occurring in some or all of these patient groups. However, this work only shows that IgE is present in the mucosa. Additional work needs to be done to establish the origin and exact role of these IgE⁺ cells. In future studies we propose to further study the mucosal physiology of noninvasive fungal sinusitis and establish the role of individual cytokines and cellular interactions in regulating the eosinophilic inflammatory cascade.

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