REVIEW

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The fungal debate: where do we stand today?*

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SUMMARY

Chronic rhinosinusitis (CRS) is an inflammatory disorder affecting the nose and paranasal sinuses. Although bacteria have long been implicated as pathogens in most forms of CRS, it has been recognized that fungi may be responsible for some forms of CRS. Recent studies have shown that under optimal conditions, fungi can be identified within the nose and paranasal sinuses of nearly every individual. Considerable controversy exists concerning the proper diagnosis of and potential overlap between 'allergic fungal rhinosinusitis' and 'chronic rhinosinusitis'. Although the disease name 'allergic fungal rhinosinusitis' is suggestive of an immunoglobulin E (IgE) mediated reaction to fungi, recent studies demonstrate the presence of elevated serum IgE levels to one fungus while another fungus is present in CRS mucin of the same individual, questioning the role of type I hypersensitivity. Several mechanisms explaining the role of fungi in the pathogenesis of CRS, all requiring additional investigations with adequate controls, have been suggested and will be reviewed. Although preliminary trials suggest a beneficial effect of topical and oral antifungal agents in the treatment of CRS patients, several double-blind placebo controlled trials do not. Presently, in the absence of convincing immunological data and evidence of clinical improvement of CRS upon therapy with antifungal agents, the case against the fungus remains unproven.

Key words: chronic rhinosinusitis, nasal polyps, fungi, eosinophils, antifungal treatment

INTRODUCTION

Chronic rhinosinusitis (CRS) is an inflammatory disease of the nose and paranasal sinuses that is present for at least 12 weeks without complete resolution and is characterized by the presence of distinctive symptoms (e.g. nasal blockage, nasal discharge, facial pain and/or reduced sense of smell) and either endoscopic signs or Computed Tomography (CT) changes characteristic of the disease[(1)]. Nasal polyposis (NP) is considered a subgroup of this disease entity. To date, the aetiology and pathogenesis of CRS are still largely unknown.

Although bacteria have long been implicated as pathogens in most forms of CRS, it has been recognized that fungi may be responsible for some forms of CRS. Fungal spores, due to their ubiquitous nature, are continuously inhaled and deposited on the airway mucosa. Although they rarely behave as pathogens in the airways of healthy individuals, they may cause human disease in some. Currently, five forms of fungal disease affecting the nose and paranasal sinuses are recognized:[1)] acute invasive fungal rhinosinusitis (including rhinocerebral mucormycosis),[2)] chronic invasive fungal rhinosinusitis,[3)] granulomatous invasive fungal rhinosinusitis,[4)] fungal ball and[5)] non-invasive (allergic) fungal rhinosinusitis (adapted from DeShazo et al[(2)]). Invasive forms of fungal rhinosinusitus are considered to be rare and generally occur in immunocompromised hosts only. Non-invasive forms of fungal rhinosinusitis, rare but slightly more common than invasive forms of fungal rhinosinusitis, include sinus fungal ball, in general affecting only one sinus, and non-invasive (allergic) fungal rhinosinusitis, affecting multiple sinuses. The latter two generally occur in immunocompetent individuals only.

Although previously considered rare, in recent years the role of non-invasive fungal pathogens in the development of CRS is increasingly debated[(3)]. Here we review the history of non-invasive fungal rhinosinusitis and focus on newer proposed roles for non-invasive fungi in the pathogenesis of CRS. Since their presence is a prerequisite in order to prove a causal relationship between fungi and CRS, the prevalence and microbiology of fungi in CRS patients will be reviewed. Special attention is given to possible immune responses that may follow from exposure to certain fungi. Finally, the role of antifungals in the treatment of CRS will be reviewed.

HISTORICAL PERSPECTIVE

In 1983, Katzenstein et al identified Aspergillus species in mucus obtained from the nose and paranasal sinuses of

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patients suffering form CRS with NP, and introduced the term "allergic Aspergillus sinusitis" because of its histopathological similarity to allergic bronchopulmonary aspergillosis (ABPA)[4]. Allergic Aspergillus sinusitis was defined as a form of CRS characterized by the presence of "allergic mucin" (thick, tenacious and darkly coloured (peanut butter like) mucus containing aggregates of necrotic eosinophils, nuclear debris, free eosinophil granules, sloughed respiratory tract epithelial cells, and Charcot-Leyden crystals within an otherwise amorphous, pale eosinophilic or basophilic mucinous background) and scattered fungal hyphae of Aspergillus species[4]. Later the disease name "allergic fungal sinusitis" (AFS) was coined, after other fungi were demonstrated to produce similar symptoms[5].

In 1994, based on clinical findings in 15 patients, Bent and Kuhn proposed 5 criteria for the diagnosis of AFS:[1] nasal polyposis,[2] allergic mucin,[3] CT findings consistent with CRS,[4] positive fungal stain or culture, and[5] type I hypersensitivity to fungi diagnosed by history, a positive skin prick test or serology[6].

In 1995, in a review of the literature of 98 AFS cases, DeShazo and Swain observed that only three fourths of patients diagnosed with AFS were atopic. Based on this observation, although to date still a point of debate, they dropped the criterion of type I hypersensitivity from the list of criteria necessary for the diagnosis of AFS[7].

Although previously considered a rare disease entity, Ponikau et al recently suggested, based on the observation that fungi are present in nearly all of their CRS patients, that AFS may be more common than previously thought. Since all CRS patients fulfilled the criteria for AFS as elaborated by DeShazo and Swain[7], Ponikau et al have suggested that the term AFS (indicating an IgE mediated response) should be replaced by "eosinophilic fungal rhinosinusitis" (EFRS) and that the term allergic mucin should be replaced by eosinophilic mucin[3].
In 2000, based on a review of the literature and new cases, Ferguson described a form of CRS histologically similar to AFS (as described by DeShazo et al[7]) except for the absence of fungal hyphae, which she called eosinophilic mucin rhinosinusitis (EMRS). It was postulated that AFS is an allergic response to fungi in predisposed individuals, while EMRS is the result of a systemic dysregulation in immunological controls[8]. Table 1 summarizes the different criteria and disease names used. Although most other authors, when discussing their patients suffering from non-invasive fungal rhinosinusitis, use the term AFS, one should bare in mind that the criteria used to define these patients differ in various studies, rendering it difficult to interpret and compare results. Both AFS and EFRS will be referred to as non-invasive fungal rhinosinusitis in the remainder of this review.

PREVALENCE AND MICROBIOLOGY OF FUNGI IN CRS

As has been elaborated above, the presence of non-invasive fungi in the nose and paranasal sinuses is required to adequately diagnose non-invasive fungal rhinosinusitis. Nevertheless, to prove the presence of fungi has been difficult. For many years, contradictory results have been published on the proportion of CRS patients having fungi in their nose and paranasal sinuses with prevalence rates ranging from 0% to 100% recently (Table 2[3,4,9-25]).

Does the collection method influence fungal yield?

Until 1999 thought of as being rare, in 1999 Ponikau et al demonstrated the presence of fungi in the nose and paranasal sinuses of nearly all CRS patients (202 of 210 (96%) consecutive CRS patients) and all healthy controls (14 of 14 (100%) healthy controls) in the US by using novel collection and culturing methods[3]. In brief, two puffs of phenylephrine hydrochloride 1% are sprayed into each nostril to produce vasoconstriction, thereby increasing nasal lumen and, consequently, yield. After 2 minutes and after taking a deep inspiratory breath, patients are instructed upon irrigation of each nostril with 20 mL of sterile saline to forcefully exhale. The return is collected in a sterile pan, rapidly transported to a specialized

Table 1. Criteria for diagnosing non-invasive fungal rhinosinusitis.

Criteria	Katzenstein[4]	Bent and Kuhn[6]	DeShazo and Swain[7]	Ponikau[3]	Ferguson[8]
Positive fungal histology or culture	X	X	X	X	
Type I hypersensitivity based on history, serology or positive skin test		X			
Allergic or eosinophilic mucin*	X	X	X	X	X
X-ray or CT-findings consistent with CRS	X	X	X	X	X
Nasal polyposis	X	X	X		X
Disease name	Allergic Aspergillus sinusitis	Allergic fungal sinusitis	Allergic fungal sinusitis	Eosinophilic fungal rhinosinusitis	Eosinophilic mucin rhinosinusitis

* Note: allergic or eosinophilic mucin is badly defined in most studies. Some studies consider a visual description adequate (i.e. peanut butter like mucinous material), while others require a histopathological description including the presence of eosinophils and/or fungal hyphae and/or Charcot-Leyden crystals.

Table 2. Prevalence of fungi.

Study	Year	Country	Collection technique	Location of collection	Detection technique	Presence of fungi in CRS patients (%)	Presence of fungi in healthy controls (%)
Katzenstein et al[4]	1983	USA	Surgically excised mucosa	Paranasal sinuses	Histological examination	6.2	
Ponikau et al[3]	1999	USA	Nasal lavage ESS guided sampling	Nasal cavity Paranasal sinuses	Culture Histological examination	96 81	100
Catten et al[9]	2001	USA	Cytology brush	Nasal septum, inferior turbinate	PCR	40	42
			Cotton swab	Nasal septum inferior turbinate	PCR	0	5.7
Taylor et al[19]	2002	USA	ESS guided sampling	Paranasal sinuses	GMS stain Fluorescein-labeled chitinase stain	76 100	
Buzina et al[23]	2003	Austria	Nasal lavage ESS guided sampling	Nasal cavity Paranasal sinuses	Culture Culture GMS stain	91.3 84.0 70.2	91.3
Braun et al[20]	2003	Austria	Nasal lavage ESS guided sampling	Nasal cavity Paranasal sinuses	Culture Histological examination	91.3 75.5	91.3
Scheuller et al[15]	2004	USA	ESS guided sampling	Middle meatus	PCR	21.1	36.8
Kostamo et al[12]	2004	Finland	Nasal lavage ESS guided sampling	Nasal cavity Paranasal sinuses	Culture PAS & GMS stain	16.7 16.7	0
Granville et al[13]	2004	USA	Surgically excised mucosa	Paranasal sinuses	GMS stain	11.7	
Weschta et al[18]	2004	Germany	Nasal lavage	Nasal cavity	Culture, fluorescence microscopy & PCR	63.3	
Jiang et al[14]	2005	Taiwan, R.O.C.	Cotton swab Nasal lavage	Middle meatus Nasal cavity	Culture Culture	11.8 49	
Kim et al[16]	2005	South Korea	Nasal lavage	Nasal cavity	PCR Culture	92.5 23.2	97.5 30.0
Polzelhl et al[11]	2005	Germany	Nasal lavage	Nasal cavity	Culture PCR	25 44	
Kennedy et al[21]	2005	USA	Nasal lavage, mucus sampling	Nasal cavity	Histological examination, culture	77.4	
Rao et al[10]	2006	USA	ESS guided sampling	Ethmoid bulla or ethmoid sinus	PCR Culture	6.5 0	0 0
Murr et al[17]	2006	USA	ESS guided brush sampling	Middle meatus	PCR	45.9	45.9
Corradine et al[22]	2006	Italy	Nasal lavage	Nasal cavity	Culture	77	

mycological laboratory and processed under laminar flow to prevent contamination. Mucolytic agents are added to release entrapped fungi by breaking apart mucus disulphide bonds. Fungi are separated from the mucus by centrifugation and are placed on various culture media to allow growth at 30°C for at least 30 days for complete recovery. By using the same nasal irrigation technique, a similar high prevalence of fungal colonization has been reported from Europe[20,23].

One could argue that it is not the improved collection technique, but rather an improvement in the detection technique that explains the increase in fungal prevalence. To answer this question, various authors have compared several collection techniques. It was shown that higher culture rates are obtained from nasal lavage specimens (49%) than from middle meatus swab specimens (11.8%)[14]. In a polymerase chain reaction (PCR)-based detection method, the cytology brush (40%) was shown to be superior to the nasal swab (0%)[9]. Although Buzina et al observed higher culture rates in nasal lavage specimens (91.3%) when compared to endoscopic sinus surgery (ESS) guided samples (84.0%)[23], suggesting that nasal lavage is superior to ESS guided sampling, specimens in this study were obtained from two different groups of individuals. In the two studies described previously, samples were obtained from the same group of individuals, thereby reducing the occurrence of selection bias. Based on the studies described above, although one could argue that not all studies are conducted properly and that the nasal lavage technique samples the nasopharynx and nasal vestibule in addition to the nasal cavity[26], the nasal lavage technique seems superior.

Does the detection method influence fungal yield?
As has been suggested above, differences in the detection method may influence fungal yield. For ESS guided samples, PCR (6.5%) has been shown to be superior to culture (0%)(10), culture (84%) has been shown to be superior to the Grocott methanamine silver (GMS) stain (70.2%)[23] and the fluorescein-labelled chitinase stain (100%) has been shown to be superior to the Grocott methanamine silver stain (76%)[19]. For specimens obtained by nasal lavage, PCR (44% and 92.5%) has been shown to be superior to culture (25% and 23.2%) by Polzehl et al and Kim et al, respectively[11,16]. Based on these comparative studies, PCR seems superior to culture, independent of the collection technique used, although one should keep in mind the near 100% yield of culture in some studies[3]. Although both PCR and culture require the presence of fungal elements, viable fungal elements are detected by culture only. Whether viability is involved in the pathogenesis of CRS is unknown. Although no other studies using the fluorescein-labelled chitinase stain have been published, the results presented by Taylor et al, demonstrating the presence of one or more fungal hyphae in the mucus of 100% of CRS specimens, are striking[19]. Since chitin is present in the cell wall of all fungi, this possibly explains the high sensitivity of this essay. Future studies are necessary to confirm these results.

Does contamination explain the high fungal yield in some studies?
It could be argued that it is the technique of fungal culture that leads to contamination and that this is why yields approach 100% in some studies. Lackner et al showed that fungi can be cultured from the nasal mucus in 20% of neonates. At the age of 2 months an individual spectrum is established and after 4 months a situation similar to the one in adults with 17 of 18 (94%) babies having a positive fungal culture has been observed[27]. The fact that most cultures from early newborns are fungus-negative strongly argues against the possibility of laboratory contamination as an explanation for the near 100% yield observed in the same population at the age of 4 months. Although laboratory contamination seems unlikely, geographic variations[28] or fungal contamination of the air in a hospital environment[29] (especially important during sampling) cannot be excluded.

Is a difference in the number and type of fungal species involved?
Since it is clear that fungi are ubiquitous in nature and equally present in the nose and paranasal sinuses of both CRS patients and healthy controls, it could be argued that it is not the presence or absence of fungi in general, but rather the presence of a certain fungal species that is relevant for the development of disease. However, in the cultures obtained by using the novel technique described by Ponikau et al[3], on average 37-40 different genera grew with 2.7-3.2 species per CRS patient and 2.3-3.1 per healthy control[3,20], with the genera Aspergillus, Penicillium, Cladosporium, Candida, Aureobasidium and Alternaria being most frequent[16,17,20,23,30] and with no significant differences in the type of fungus being present.

Is it the fungal (allergen) load that is relevant for the development of disease?
It could be argued that it is not the presence or absence of a certain fungus, but rather the fungal load or the amount of fungal allergen that is critical for the development of disease. Schueller et al, in a recent prospective study investigating 19 CRS patients and 19 non-CRS patients, failed to demonstrate a significant difference in the amount of fungal DNA present in both groups, rendering it unlikely that it is the quantity of fungal elements that is critical for the development of CRS[15]. Whether an increase in fungal allergen content is involved in the pathogenesis of CRS is to date still a point of debate. For Alternaria, it has been shown that allergen content can vary between strains of the same fungus and between components of the same isolate (spores versus hyphae). Germination of Alternaria spores significantly increases the number of spores releasing allergen, including the major allergen Alt a 1. Recently, it has been suggested that germination of fungal spores takes place in the respiratory tract and especially in mucus. Upon germination, greater quantities of allergen are released[31]. As the presence of mucus precedes the increase in

allergen release, it is unlikely that it is the fungus that is the causative pathogen in CRS. Based on these observations, it is more likely that fungi are involved in CRS exacerbations in susceptible individuals.

Summary

Fungi can be detected in the nose and paranasal sinuses of all CRS patients and all healthy controls. Improved collection and detection techniques may have played an important role in the increase in fungal yield observed recently. No differences in fungal load and fungal species have been consistently demonstrated between CRS patients and healthy controls. Whether differences in fungal allergen content play a role in the development of non-invasive fungal rhinosinusitis in some individuals is to be elucidated.

HUMORAL IMMUNE RESPONSES TO FUNGI

Is type I hypersensitivity to fungi involved in the pathogenesis of CRS?

For many years, an immunoglobulin(Ig) E (IgE)-mediated systemic fungal allergy (as demonstrated by the presence of elevated levels of fungal specific IgE or a positive skin prick test to common airborne fungi) has been said to drive the pathologic process characteristic of non-invasive fungal rhinosinusitis. Recently, it has been suggested that non-invasive fungal rhinosinusitis is more common than hitherto expected based on the recent finding that fungi are present in the nose and paranasal sinuses of nearly all CRS patients. If fungal allergy is indeed necessary to adequately diagnose non-invasive fungal rhinosinusitis, as is debated by various authors, one should be able to distinguish diseased patients from healthy controls based on elevated levels of fungal specific IgE or a positive skin prick test to common airborne fungi.

Various authors have studied sensitization rates to fungi in CRS patients, demonstrating values ranging from 18% to 46%[3,19,32,33]. Some authors reported no difference in sensitization rates to fungi between CRS patients and healthy controls[3], while others observed higher levels of fungal specific IgE in patients with CRS with eosinophilic mucin (with or without fungi) when compared to healthy controls[34]. Although higher in CRS patients with eosinophilic mucin, it should be noted that no significant differences were observed between this group of CRS patients and a group of patients suffering from allergic rhinitis without sinus involvement but with a proven allergy to fungi[34]. As is true for ABPA, a positive immediate skin test is not specific for ABPA and reflects the presence of antigen-specific IgE (as other atopic subjects may also be sensitized to Aspergillus *fumigatus*) without any evidence of the parenchymal features that are required to accurately diagnose ABPA[35]. Thus, the presence of fungal antigen-specific IgE does not distinguish non-invasive fungal rhinosinusitis patients from other CRS patients.

One may suggest that it is not an allergy to fungi in general, but an allergy to a certain fungus in particular that is important for the development of non-invasive fungal rhinosinusitis. Although studying only a small number of fungi (Alternaria *alternata*, Aspergillus *fumigatus*, Cladosporium *herbarum* and Penicillium *notatum*), Shin et al observed similar serum levels of IgE to various common airborne fungi in 18 patients with CRS and 15 healthy controls. IgE antibodies to Alternaria *alternata*, Aspergillus *fumigatus*, Cladosporium *herbarum* and Penicillium *notatum* were present in less than 30% of the patients despite the presence of Alternaria protein in nasal secretions of all tested (n=9) CRS patients[36], suggesting that it is not an allergy to a specific fungus that is important for the development of CRS. Since recent observations by Pant et al demonstrate that some patients with non-invasive fungal rhinosinusitis do not have an allergy to the fungus identified in their eosinophilic mucin but may have elevated IgE levels to other fungi, it should be questioned whether the presence of fungal allergy is relevant for the development of CRS[34]. Thus, as has been suggested by DeShazo et al, type I hypersensitivity to fungi may not be of central pathogenic importance for the development of non-invasive fungal rhinosinusitis[7]. Based on the results described above, the assumption of a unique pathogenic role of fungal allergy in non-invasive fungal rhinosinusitis should be questioned. Its presence more likely represents concurrent fungal allergy in most CRS patients.

Is there a role for fungal specific IgG?

Since sensitization rates to fungi are similar, a differential immunological response to fungi involving IgG (and not IgE) has been suggested. Shin et al recently demonstrated a significant increase in IgG levels to Alternaria *alternata*, Aspergillus *fumigatus*, Cladosporium *herbarum* and Penicillium *notatum* in CRS patients in comparison to healthy controls[36]. Intriguingly and in line with these results, Pant et al demonstrated high levels of fungal-specific IgG1 and IgG3 isotypes to Alternaria *alternata* and Aspergillus *fumigatus* in CRS patients with eosinophilic mucin (n = 30) when compared to healthy controls[34]. When compared to allergic rhinitis patients (with a proven allergy to fungi) or CRS patients without eosinophilic mucin, Pant et al observed that fungal-specific IgG3 is characteristic of CRS patients with eosinophilic mucin, regardless of the presence of fungi or of systemic fungal allergy[34]. IgG2 and IgG4 are elevated in CRS patients with eosinophilic mucin compared to healthy controls, but levels are not significantly greater when compared to allergic rhinitis patients or CRS patients without eosinophilic mucin. Human IgG2 and IgG4 isotypes, which do not activate complement or bind Fc receptors well, were recently shown to protect BALB/c mice against infection with the fungus Cryptococcus *neoformans*. Surprisingly, human IgG1 and IgG3 isotypes, which activate complement and bind all three classes of Fc receptors, functions classically viewed to be essential for antibody mediated protection against infections, were not protective and IgG1 actually reduced survival[37]. To date, other than being a biologic marker for CRS patients with eosinophilic mucin, the pathogenic significance of fungal-specific IgG3 has not been determined.

Summary
To date, no difference in the prevalence of fungal allergy has been observed between CRS patients and healthy controls. Since CRS patients may have elevated IgE levels to one fungus while another fungus is present in their eosinophilic mucin, it should be questioned whether a type I hypersensitivity to fungi is relevant for the development of non-invasive fungal rhinosinusitis. Whether fungal specific IgG (especially IgG1 and IgG3) is involved in the pathogenesis of CRS requires additional research.

CELLULAR IMMUNE RESPONSE TO FUNGI IN CRS

The normal immune response varies with respect to the fungal species, the morphotype encountered and the anatomical site of interaction. Whereas yeasts and spores are often effectively phagocytosed, the larger size of hyphae precludes effective ingestion and requires interaction with different inflammatory cells[38]. Although neutrophils, macrophages and monocytes are important antifungal effector cells, most research in non-invasive fungal rhinosinusitis has focused on the role of the eosinophil. Traditionally, eosinophils are thought of as major effector cells in allergic inflammation. They synthesize, store and release a wide range of pro-inflammatory mediators, including four cationic proteins (major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN), eosinophil peroxidase (EPO)) and up to 23 cytokines and growth factors. Besides being involved in allergic inflammation, eosinophils do also play an important role in non-allergic inflammation and host immunity to helminth infections[39].

Is the presence of fungi related to tissue eosinophilia?

Although their observation is questioned by some authors, Ponikau et al recently observed that, in addition to fungi, eosinophils are present in tissue specimens of nearly all CRS patients[3]. Eosinophilic inflammation was shown to be heterogeneous in any given tissue specimen with areas of abundant eosinophilia and areas without eosinophils[40]. Careful collection of tissue specimens with mucus remaining attached to the harvested tissue has been suggested to be necessary to determine the extent of tissue eosinophilia and the localization and degranulation pattern of eosinophils[3,20,40,41]. Careful evaluation of numerous sites of one single sample seems crucial to avoid the so-called false-negatives that explain the absence of eosinophils in the CRS specimens studied by others[20,40]. Although fungi, due to their ubiquitous nature, are present in both CRS patients and healthy controls, tissue eosinophilia has been observed in CRS patients only.

Is it an aberrant eosinophilic immune response to ubiquitous airborne fungi that distinguishes CRS patients from healthy controls?

By using a modified Boyden chamber, Wei et al studied the extent of eosinophil migration of eosinophils from both CRS patients and healthy controls in the presence of CRS nasal mucin and CRS nasal tissue, demonstrating a concentration-dependent increased migration of eosinophils towards both CRS nasal mucin and CRS nasal tissue extracts[42]. The percentage of migration was consistently higher for eosinophils obtained from patients with CRS. Nine out of 10 CRS patients, however, were diagnosed with asthma and four out of 10 patients were atopic. Although Wei et al observed greater (and due to group size not significant) eosinophil migration in atopic patients in response to both nasal mucin and nasal tissue extracts when compared to non-atopic patients, the role of asthma has not been taken into account. Several studies have demonstrated that eosinophils from subjects with asthma (both allergic and non-allergic asthma) exhibit a primed phenotype that is likely the consequence of eosinophil interaction with cytokines in the peripheral blood, resulting in increased migration, adhesion and degranulation properties[43-45]. Based on these data, it remains unclear whether the observed increased migration of CRS eosinophils is the consequence of the disease CRS itself or that it is related to the underlying asthma and/or atopy.

If one assumes that ubiquitous airborne fungi induce tissue eosinophilia in susceptible individuals, is a T-cell dependent mechanism involved in eosinophil recruitment?

If fungi are able to trigger inflammatory cells to initiate a complex localized eosinophilic reaction in susceptible individuals, one may postulate that this process involves T-cells reacting to fungal antigens by producing a T helper cell 2 (T_H2) dominated cytokine profile, including interleukin-5 (IL-5) and IL-13. IL-5 is the most important cytokine for inducing eosinophilic inflammation, stimulating eosinophil production, chemotaxis, survival and activation[46]. IL-13 induces the expression of vascular cell adhesion molecule 1 (VCAM-1) involved in selective eosinophil migration from the vasculature into the tissue[46].
If one assumes that fungi are able to initiate an eosinophilic reaction in CRS patients but not in healthy controls, it could be that patients and controls differ in their T-cell response to fungal antigens. Although Shin et al observed no significant differences in the proliferation of peripheral blood mononuclear cells (PBMCs) upon culture with extracts from 4 common airborne fungi (Alternaria *alternata*, Aspergillus *fumigatus*, Cladosporium *herbarum* and Penicillium *notatum*) between CRS patients and healthy controls, striking differences in PBMC cytokine responses were reported. When cultured with Alternaria *alternata* extract, 89% of the PBMCs obtained from CRS patients produced significantly more IL-5 and IFN-γ when compared to healthy controls. In addition, some patients produced more IL-5 in response to Aspergillus *fumigatus* (22%) and Cladosporium *herbarum* (33%). PBMCs from all CRS patients produced IL-13 upon culture with Alternaria *alternata*, Aspergillus *fumigatus* and Cladosporium *herbarum* extracts[36]. Unfortunately, although 61% of the patients demonstrated increased levels of IgE to common aeroallergens and 78% of the patients suffered from bronchial

asthma, no adequate controls (i.e. CRS patients without allergy and asthma, CRS patients with allergy but without asthma, CRS patients without allergy but with asthma and allergic rhinitis patients) were included in this study rendering the interpretation of these results difficult. Although it is mentioned that cytokine responses of PBMCs obtained from CRS patients with or without atopy are similar, the role of asthma has not been taken into account. As was shown by Haselden et al, PBMCs from asthmatic subjects (both allergic and non-allergic) produce more IL-5 in response to allergen when compared to allergic rhinitic subjects and healthy controls[47].

Are mechanisms other than T-cell dependent pathways involved in eosinophil activation?

Although airborne fungi may contribute to the development and exacerbation of (allergic) disease via T_H2 cell-dependent, antigen-mediated immune responses with tissue eosinophilia as the ultimate result, recent studies indicate that fungi may also activate eosinophils directly. Inoue et al recently demonstrated that Alternaria *alternata*, but not IL-5, is able to induce eosinophilic IL-8 synthesis and surface expression of CD11b (a β2-integrin that is an activation marker for eosinophils[48]) and CD63 (also known as lysosome-associated membrane protein-3, a component of eosinophil granule membranes[49]) on eosinophils obtained from healthy volunteers and patients with allergic rhinitis or asthma[50]. In addition, although the molecular mechanisms for exocytosis of eosinophilic cationic granules are incompletely understood, it was demonstrated that intracellular and extracellular Ca^{2+} are key factors in Alternaria *alternata* and Penicillium *notatum* induced eosinophil degranulation[50]. For Alternaria *alternata* it was shown that the observed increase in intracellular Ca^{2+} is mediated by pertussis toxin sensitive G protein-coupled receptors[50]. Upon stimulation with Alternaria *alternata*, an increased degranulation propensity was observed for eosinophils obtained from all tested individuals. However, eosinophils from patients with asthma or allergic rhinitis released 70% more EDN upon stimulation with Alternaria *alternata* than healthy controls[50]. Whether this increased degranulation propensity of eosinophils is a direct effect of Alternaria *alternata*, the consequence of a primed phenotype of eosinophils or both is unclear. Unfortunately, eosinophils from CRS patients were not included, rendering it unclear whether similar observations are true for CRS eosinophils.

Summary

Eosinophils are present in nearly all CRS tissue specimens. Since tissue eosinophilia is heterogeneous in any given tissue specimen, careful evaluation of numerous sites of one single sample seems crucial to avoid so-called false-negatives. Whether it is the presence of fungi that causes the tissue eosinophilia in susceptible individuals requires additional research with adequate controls. If so, both T-cell dependent and/or T-cell independent pathways may be involved.

THE ROLE OF NASAL MUCOSA

The epithelium is the point of first contact for airborne particles (including fungi) and as such constitutes the interface between the external environment and the internal milieu of the nose. Under normal circumstances the epithelium forms a highly regulated and almost impermeable barrier through the formation of tight junctions, cell-cell and cell-extracellular matrix interactions. Recently, it has become clear that it is the epithelium that orchestrates the inflammatory and remodelling responses of the airway (for review see Hackett and Knight[51]). Airway remodelling is a pathologic process characteristic of both asthma and CRS and involves epithelial metaplasia and damage, thickening of the subepithelial basal lamina, increase in the number of myofibroblasts, hypertrophy and hyperplasia of airway smooth muscle, mucus gland hyperplasia, angiogenesis and altered deposition and composition of extracellular matrix proteins (for review see Busse et al and Pawankar et al[52,53]).

If fungi are causative of CRS, is the epithelial damage observed in CRS specimens the result of a T-cell dependent pathway or may other mechanisms be involved?

Epithelial damage as observed in CRS specimens may be the result of a T_H2 cell-dependent, antigen-mediated immune response that ultimately results in the release of toxic eosinophilic granules. Eosinophilic cationic proteins are toxic to respiratory epithelial cells and release has been shown to result in ciliostasis, desquamation and destruction in vitro[54]. Recent observations suggest that ubiquitous airborne fungi, especially Alternaria *alternata* and Aspergillus *fumigatus*, produce proteases that bind to protease-activated receptors (PARs) expressed on epithelial cells, airway cells, leukocytes and blood vessels thereby activating intracellular signalling pathways that give rise to multiple responses, including the production of chemokines, cytokines, eicosanoids, and metalloproteinases, that may ultimately result in the disruption of the tight junctions that bind epithelial cells to each other and to the basement membrane[55,56]. Compared to the proteases from Aspergillus *fumigatus* and Cladosporium *herbarum*, proteases from Alternaria *alternata* are most potent in inducing the production of inflammatory cytokines (IL-6, IL-8) from primary nasal epithelial cells[55]. Although these observations suggest that responses to Alternaria *alternata* may differ from those to other fungi, this does not explain the difference in response observed between CRS patients and healthy controls. Whether genotypic differences in PAR expression or other factors that enhance the action of PARs in response to stimulation by fungal proteases are involved, remains unclear.

Although fungus activated PARs may play a role in airway remodelling, one may postulate that airway remodelling is a separate process in CRS. A recent biopsy study in asthmatic children demonstrated collagen deposition and fibroblast proliferation prior to eosinophil infiltration of the lung, suggesting

that the asthmatic epithelium functions abnormally even in the absence of inflammation[57]. As epithelial damage is observed in nearly all CRS patients as well (but not in allergic rhinitis patients who share many characteristics with both diseases including increased mucus production, tissue eosinophilia and, due to their ubiquitous nature, the presence of fungi), it may well be that similar intrinsic epithelial abnormalities explain the development of CRS in susceptible individuals. In this context, extramucosal fungi more likely act as entrapped passengers or as catalysts.

Summary

Airway remodelling is a pathologic process characteristic of both asthma and CRS. As has been demonstrated for asthma, airway remodelling and inflammation may occur independently. Whether fungi are involved in airway remodelling (either as a result of eosinophil degranulation or due to activation of PARs) remains to be elucidated.

ANTIFUNGAL TREATMENT

If the inflammation observed in CRS patients is the result of an immune reaction to fungi, reducing the presence of this inflammatory trigger might improve the course of the disease[3]. Ideally, treatment should eliminate the fungus without causing harm to the host. In 1996, 22 fungal cultures grown from 15 AFS patients were studied by Bent and Kuhn for in vitro susceptibility to five common antifungal drugs (ketoconazole, amphotericin B, itraconazole, nystatin and fluconazole). Ketoconazole and amphotericin B were shown to be most effective, independent of the fungal organism tested[58]. Amphotericin B is active against most fungi frequently identified within the nose and paranasal sinuses[59]. Despite its clinical effectiveness, the use of systemic amphotericin B is limited by adverse systemic reactions, including fevers, chills, nausea, diarrhoea and neutropenia as well as damage to kidneys and liver. Topical treatment may have the advantage in that high concentrations may be achieved locally without causing major systemic side effects.

Although the injectable formulation of amphotericin B carries US Food and Drug Administration-approved labelling solely for intravenous administration, several alternative routes of administration that use the injectable formulation have been reported including the administration of amphotericin B into the pleural cavity[60], bladder[61-63], synovial joints[64] and peritoneal space[65].

Is topical amphotericin B effective in the treatment of CRS?

Various studies investigating the effectiveness of topical antifungals in CRS have been published in recent years (Table 3). In 2002, The Mayo Clinic treated 51 patients with CRS with intranasal amphotericin B in an open-label study[32]. After 3 months of treatment 38 (75%) of 51 patients showed improvement of sinusitis symptoms and nasal endoscopy scores. Although 12 of 13 patients showed substantial improvement in maxillary sinus CT findings, it should be noted that a reduction in mucosal thickening on a coronal CT of a maxillary sinus correlates poor with symptom scores[66]. In addition, since only 13 of 51 patients were selected for a second sinus CT-scan, selection bias is implied. Since no placebo group was included, it remained unclear whether it is the amphotericin or the nasal lavage that was effective.

In the same year, a second uncontrolled trial treated 74 patients with intranasal amphotericin B for a period of 4 weeks studying the effect of amphotericin B on nasal polyp stage[67]. Total disappearance of nasal polyps was observed in 29 (39%) of 74 patients, all having polyps confined to the middle meatus (62% cured) or extending just beyond the middle turbinate (42% cured). Of the patients with polyps filling up the complete nasal cavity, none was cured, suggesting that polyp stage is a critical determinant of treatment outcome. Besides polyp stage, it was shown that previous ESS resulted in better response rates. This may be due to a better penetration of the drug in surgical cavities. Again, no placebo group was included.

When subjected to a randomized, double-blind, placebo-controlled trial, involving 78 patients with CRS, no significant benefit from long term use (8 weeks) of an amphotericin B nasal spray was observed [18]. In total, 60 of the 78 enrolled patients finished the study per protocol. Although penetration has been shown to be inferior to nasal lavages, especially when used in the kneeling position[68], symptom scores were distinctly worse upon therapy with an amphotericin B nasal spray. All other investigated parameters, including CT-scan scores for maxillary sinus opacification, quality of life scores, endoscopy scores and presence of fungal elements in nasal lavages, did not differ between the two treatment groups. Importantly, none of the investigated outcome variables improved in the subgroup of patients in whom fungal elements had been detected before but not after treatment with amphotericin B, questioning the hypothesis that elimination of the supposed causative agent improves the course of the disease.

A second randomized, double-blind placebo-controlled single center study used amphotericin B to treat 30 randomly selected patients with CRS[25]. Only 24 of the 30 enrolled patients completed the 6 month treatment period. Although a reduction of 8.8% in the amount of inflammatory mucosal thickening on CT-scan (the primary outcome measure) was reported as clinical significant, it is questionable whether this minimal reduction in inflammatory mucosal thickening on CT-scan (known to correlate poorly with clinical symptoms[66]) is clinically relevant. Laboratory evaluation showed decreased EDN levels (but not decreased levels of IL-5, Alternaria protein and eosinophils) upon treatment with amphotericin B. Although nasal endoscopy scores improved significantly, symptom scores (SNOT-20) did not.

Contrasting with the previous study and in line with the results presented by Weschta et al[18], a recent large European double-blind placebo-controlled multicenter study, treating 116 patients with CRS with amphotericin B nasal lavages or place-

Table 3. Studies on topical and oral antifungals in CRS patients.

Author	Year	Country	Active drug (n)	Placebo (n)	Drug name	Solvent	Dose	Duration	Method	Study design	Level of evidence	Outcome
Ponikau et al[32]	2002	US	51	0	Amphotericin B	Sterile water	100 µg/mL 20 mL twice daily each nostril	3-17 months	Nasal lavage	Non-placebo controlled single center study	Level III	Positive
Ricchetti et al[67]	2002	Switzerland	74	0	Ampho-moronal (Bristol-Myers Squibb)	Sterile water	1:1000 20 mL twice daily each nostril	4 weeks	Nasal lavage	Non-placebo controlled single center study	Level III	Positive
Weschta et al[18]	2004	Germany	28	32	Amphotericin B (Bristol-Myers Squibb)	5% glucose (sodium phosphate buffered)	3 mg/mL 200µL four times daily each nostril	8 weeks	Nasal spray	Randomized placebo-controlled double-blind single center study	Level Ib	Negative
Ponikau et al[25]	2005	US	10	14	Amphotericin B	Sterile water	250 µg/mL 20 mL twice daily each nostril	6 months	Nasal lavage	Randomized placebo-controlled double-blind single center study	Level Ib	Positive (CT) & negative (symptoms)
Kennedy et al[21]	2005	US	25	28	Terbinafine (Novartis Pharma AG)	Not applicable	625 mg/day	6 weeks	Oral	Randomized placebo-controlled double-blind single center study	Level Ib	Negative
Ebbens et al[33]	2006	Netherlands, UK, Spain, Belgium	59	57	Amphotericin B (Bristol-Myers Squibb)	Glucose 2.5%	100 µg/mL 20 mL twice daily each nostril	13 weeks	Nasal lavage	Randomized placebo-controlled double-blind multicenter study	Level Ib	Negative

bo, failed to show improvement in symptom scores as assessed with the visual analogue scale (VAS), the amount of nasal obstruction as assessed by peak nasal inspiratory flow (PNIF), nasal endoscopy scores, polyp scores and quality of life scores (rhinosinusitis outcome measure 31 (RSOM-31), short form 36 (SF-36)) after 3 months of treatment, suggesting that topical therapy with amphotericin B does not result in clinically relevant results[33].

Are oral antifungals effective in the treatment of CRS?

Several anecdotal uncontrolled reports have been published describing the effectiveness of oral antifungal agents in the treatment of CRS. Rains and Mineck, in a 12-year retrospective chart review of 139 patients meeting the AFS criteria of fungal atopy, characteristic radiographic findings, eosinophilic mucin, nasal polyposis, and a positive fungal culture or stain, showed that oral itraconazole in combination with topical and oral corticosteroids may result in a reduction of revision surgery[69]. Questions arise as to whether the observed results are caused by a steroid potentiating effect of oral itraconazole or the result of its antifungal action.

In contrast with the study by Rains et al[69] and in line with previous results on topical antifungal treatment[18,33], Kennedy et al failed to show significant improvements in total opacification score (CT sinus), total obstruction score (CT sinus), total rhinosinusitis disability index (RSDI) score and the functional, physical and emotional domains of the RSDI upon treatment with high-dose oral terbinafine (625 mg/day) for a period of 6 weeks in a double-blind placebo-controlled trial including 53 CRS patients[21]. Terbinafine levels were measured in post-treatment sinus biopsies of selected patients, demonstrating that terbinafine levels were well within minimum inhibitory concentration (MIC) ranges for isolates thought to play a role in CRS[21]. Although tissue terbinafine levels were well within MIC ranges for fungal isolates thought to play a role in CRS, questions arise as to whether tissue bioavailability of oral terbinafine is similar to mucus bioavailability. As has been suggested by Ponikau et al[3], fungi reside extramucosally outside the range of the drug circulation. In order to produce an effect, a systemic antifungal must be secreted in sinus mucus, a phenomenon that has not been documented and may not occur.

Is topical amphotericin B safe to use?

Although the advantages are clear, topically applied drugs may have cytotoxic effects. To rule out this possibility, Hofer et al studied the effect of topical amphotericin B on ciliary beat frequency (CBF). When diluted in saline, no effect of amphotericin B (0.1 mg/mL) on CBF was observed. When diluted in distilled water, CBF was irreversibly lowered to about 50%, suggesting that physiologic solvents should be used[70]. Confirming the findings by Hofer et al, Gosepath et al observed minimal ciliotoxicity upon treatment with low concentrations of amphotericin B (2.5%, 5%). After increasing the concentration to a 10% solution, CBF dropped[71]. Although, based on these results, cytotoxicity in the described dosing regimens is unlikely, the effect of repeated dosing over time on CBF is unknown.

If effective, how does topical amphotericin B exert its effect?

Topical amphotericin B treatment has been suggested to reduce fungal load, thereby reducing the inflammatory

response in the nose and paranasal sinuses and, ideally, resulting in the resolution of CRS. Recent data suggest that amphotericin B, besides having an anti-fungal effect, may have a direct cytotoxic effect on nasal polyp epithelial cells. Amphotericin B is a sterol-binding agent with high affinity for ergosterol (the dominant fungal sterol) and low affinity for cholesterol (the mammalian sterol) and is known to modify cell membrane structure by forming aqueous pores in lipid membranes, resulting in an increase in membrane permeability to small ions (inward leak of Na^+, outward leak of K^+) and, consequently, activation of the Na^+ K^+ -ATPase pump and modifications in transepithelial resistance. By treating human nasal polyp epithelial cells with amphotericin B (50 M, 4 hours daily for 5 days), Jornot et al observed an increase in cell permeability and, as a consequence, a disruption of cell monolayer integrity (as demonstrated by a 60% drop in transepithelial resistance). In addition, a significant loss in cell number and expression of the tight junction protein occludin was demonstrated using immunofluorescence microscopy. The integrity of turbinate epithelial cells, however, was conserved (i.e. no change in transepithelial resistance), suggesting a differential effect on both cell types[72]. For turbinate epithelial cells, Jornot et al recently observed that amphotericin B treatment results in a decrease in transepithelial potentials, short-circuit currents, and Na^+ absorption. This inhibition of Na^+ transport was associated at first with decreased apical sodium channel (EnaC) activity and followed by a decrease in basolateral Na^+ K^+ -ATPase pump activity and K^+ conductance, possibly reflecting a feedback mechanisms that aims to limit cellular Na^+ overload and K^+ depletion subsequently to formation of amphotericin B pores in the cell membrane[73]. Whether an aberrant feedback mechanism results in the disruption of cell monolayer integrity and cell death in nasal polyp epithelium remains unclear.

In addition to a possible cytotoxic effect on epithelial cells of CRS patients with nasal polyps, it has been suggested that amphotericin B may have anti-inflammatory properties. However, a 4-week treatment regimen with topical amphotericin B (50 or 100 mg/L, 10 mL two times daily), was shown not to result in a significant reduction in IL-5, IL-8, IFN-γ and RANTES (regulated upon activation of normal T-cell expressed and secreted) levels [74]. In addition, an 8-week treatment regimen with a topical amphotericin B spray (3 mg/mL, 200 L per nostril, 4 times daily) was shown not to result in a significant reduction in ECP and tryptase levels in nasal lavage fluid from patients with CRS. Neither topical amphotericin B therapy nor fungal state before and after treatment had any significant influence on ECP and tryptase levels, although a slight improvement in ECP level was observed in those patients with successful elimination of fungus when compared to those patients with persistent fungus[75].

Summary

Although safe to use and despite initial evidence of benefit in two uncontrolled studies[32,67], three subsequent double-blind placebo controlled studies either failed to show clinical benefit[18,33] or showed, at best, only modest benefit of topical amphotericin B treatment in patients with CRS[25]. Although therapeutic effects of amphotericin B are said to result from its antifungal effect, therapeutic effects may also result from a selective cytotoxic effect on CRS epithelium. Topical amphotericin B is unlikely to have anti-inflammatory properties. Similar to topical antifungals, no clear evidence exists justifying the routine use of oral antifungal agents in the treatment of CRS patients[21].

CONCLUSIONS AND FUTURE DIRECTIONS

The role of fungi in CRS remains to be defined. Although different studies have agreed that both fungi and eosinophils can be detected in nearly all CRS patients, fungi are present in healthy controls as well. Currently, there are more questions than answers concerning the cause of CRS and the role of fungi. Recent studies suggest that many mechanisms may be involved by which fungi can cause disease in some individuals. Future studies will have to clarify the role of fungi in CRS, which fungal organisms or which components of fungal organisms may be pathogenic, which individuals are susceptible, and what exactly characterizes the immunologic response to fungi that results in the development of CRS. Presently, in the absence of convincing immunological data (including studies with adequate controls) and evidence on clinical improvement of CRS upon therapy with both topical and oral antifungal agents, the case against the fungus remains unproven.

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