The mold conundrum in chronic hyperplastic sinusitis

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Purpose of review

To review the literature on the role of fungi in chronic rhinosinusitis pathogenesis and the effect of antifungal drug therapy (both topical and oral) and antifungal immunotherapy.

Recent findings

This paper reviews the most recent articles investigating the role of fungi in CRS pathogenesis. Besides possible aberrant innate and adaptive anti-fungal immune responses and fungus anti-host effects, which all may explain disease development, the effect of antifungal drug therapy (both topical and oral) and antifungal immunotherapy are reviewed.

Summary

The role of fungi in CRS remains to be defined. Although different studies have agreed that fungi can be detected in the nose and paranasal sinuses of nearly all CRS patients, they 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 there are many mechanisms by which fungi can exert an effect on sinus mucosa in susceptible individuals. Future studies will have to clarify the role of fungi in CRS, which fungal organisms, if at all, may be pathogenic, and what exactly characterizes the immunologic response to fungi that may potentially results in the development of disease. Presently, in the absence of convincing immunological data and evidence for clinical improvement of CRS upon therapy with antifungal agents, the case against the fungus remains unproven.

Keywords

chronic rhinosinusitis, nasal polyps, allergic fungal sinusitis, fungi, pathogenesis, antifungals, immunotherapy

Number of words

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{Fokkens, 2007 13 /id;Fokkens, 2007 16 /id}. 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. 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 occasionally be the cause of disease in some.

In 1983, Katzenstein et al identified non-invasive *Aspergillus* species in thick, tenacious, dark-coloured eosinophilic mucus (so called eosinophilic or allergic mucin) obtained from the nose and paranasal sinuses of patients suffering from CRS with nasal polyps and introduced the term “allergic *Aspergillus* sinusitis” because of histopathological similarities with allergic bronchopulmonary aspergillosis{Katzenstein, 1983 246 /id}. Later the disease name “allergic fungal sinusitis” (AFS) was coined after other non-invasive fungi were demonstrated to produce similar symptoms{Robson, 1989 247 /id}. As clinical evidence of AFS accumulated, controversy regarding its definition (should fungal allergy be present?), prevalence, and disease mechanisms emerged{Bent, 1994 248 /id;deShazo, 1995 238 /id}. When Ponikau et al demonstrated the presence of both fungi and eosinophils in the nose and paranasal sinuses of nearly all CRS patients by using

novel collection, culturing and histology techniques, thus suggesting that the majority of CRS patients actually suffers from AFS, the discussion about the definition, prevalence and disease mechanisms of AFS was boosted{Ponikau, 1999 20 /id}.

PREVALENCE AND MICROBIOLOGY OF FUNGI

Prevalence of fungi: ubiquitous in both CRS patients and healthy controls

The presence of non-invasive fungi in the nose and paranasal sinuses is required for the diagnosis of AFS. Nevertheless, to prove their presence has been difficult. For many years, contradictory results have been published with prevalence rates ranging from 0% to 100% in both CRS patients and healthy controls{Katzenstein, 1983 246 /id;Ponikau, 1999 20 /id;Catten, 2001 251 /id;Rao, 2006 24 /id;Polzehl, 2005 252 /id;Scheuller, 2004 257 /id;Kim, 2005 258 /id;Murr, 2006 256 /id;Weschta, 2004 5 /id;Taylor, 2002 245 /id;Kennedy, 2005 81 /id;Buzina, 2003 21 /id;Ponikau, 2005 8 /id;Jiang, 2005 255 /id;Hafidh, 2007 295 /id;Tosun, 2007 293 /id;Aydil, 2007 294 /id;Liang, 2008 117 /id;Kostamo, 2004 253 /id;Granville, 2004 254 /id;Gosepath, 2004 261 /id;Braun, 2003 79 /id;Corradini, 2006 260 /id} (a comprehensive overview of all studies was recently published in *Rhinology*{Ebbens, 2007 304 /id}). As was suggested by Ponikau et al{Ponikau, 1999 20 /id}, differences in collection and detection techniques may explain the observed differences in fungal yield. Of all the collection techniques, the nasal lavage technique is considered to be superior{Catten, 2001 251 /id;Buzina, 2003 21 /id;Jiang, 2005 255 /id}. Although Ponikau et al describe a prevalence rate of 100% upon culture in their study using novel collection and detection techniques{Ponikau, 1999 20 /id}, most authors agree that PCR is superior to both culture and Grocott methanamine silver stains in

detecting fungal elements{Rao, 2006 24 /id;Buzina, 2003 21 /id;Polzehl, 2005 252 /id;Kim, 2005 258 /id;Aydil, 2007 294 /id}. Although no other studies using the fluorescein-labelled chitinase stain have been published to date, the detection of one or more fungal hyphae in 100% of CRS mucus specimens is striking and warrants future research{Taylor, 2002 245 /id}.

Microbiology of fungi: no difference between CRS patients and healthy controls

Since fungi are ubiquitous in nature and equally present in the nose and paranasal sinuses of both CRS patients and healthy controls, one could argue that it is not the presence or absence of fungi, but rather the fungal species or fungal load that is relevant for disease development. However, in cultures collected via the novel technique described by Ponikau et al{Ponikau, 1999 20 /id}, 37-40 different genera grew with 2.7-3.2 species per CRS patient and 2.3-3.1 per healthy control with the genera *Aspergillus, Penicillium*, *Cladosporium, Candida, Aureobasidium* and *Alternaria* appearing most frequently and with no significant differences between the two groups{Ponikau, 1999 20 /id;Buzina, 2003 21 /id;Kim, 2005 258 /id;Murr, 2006 256 /id}. In addition, there were no differences in the amount of fungal DNA present in tissue specimens obtained from CRS patients and healthy controls{Scheuller, 2004 257 /id}, rendering it unlikely that fungal species and fungal load play a role in disease development. Whether an increase in fungal *allergen* content is involved in CRS pathogenesis remains unclear.

TYPE I HYPERSENSITIVITY TO FUNGI

For many years, an immunoglobulin E (IgE)-mediated systemic fungal allergy has been thought to drive the pathologic process characteristic of AFS. If fungal allergy is necessary to adequately diagnose AFS one should be able to distinguish diseased patients from healthy controls based on elevated levels of fungus 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 75%{Ponikau, 1999 20 /id;Taylor, 2002 245 /id;Ponikau, 2002 244 /id;Ebbens, 2006 299 /id}, with no significant differences between those patients with fungi and those patients without fungi in their nose and paranasal sinuses{Tosun, 2007 293 /id}. Compared to healthy controls, some authors report similar sensitization rates in all CRS patients{Ponikau, 1999 20 /id}, while others report higher levels of fungus specific IgE in those CRS patients with eosinophilic mucin (with or without fungi){Pant, 2005 268 /id}. Although higher in CRS patients with eosinophilic mucin, no significant differences were observed between this group of patients and group of patients suffering from allergic rhinitis with proven allergy to fungi but without sinus involvement{Pant, 2005 268 /id}. In addition, Shin et al recently showed that IgE levels to various common airborne fungi (*Alternaria, Aspergillus, Cladosporium* and *Penicillium)* are similar in 18 CRS patients and 15 healthy controls, rendering it unlikely that an allergy to a specific fungus is involved{Shin, 2004 242 /id}. Since Pant et al recently demonstrated that some patients with CRS do not have an allergy to the fungus that is present in their eosinophilic mucin but may have elevated IgE levels to other fungi, one should question whether the presence of type I hypersensitivity to fungi is relevant for disease development{Pant, 2005 268 /id}. Most likely, the presence of type I hypersensitivity to fungi represents concurrent fungal allergy in the majority of CRS patients.

NASAL HOST DEFENSE AGAINST FUNGI

The nasal mucosa 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{Vroling, 2008 317 /id}. Upon exposure, the innate immune system ensures the initial defense against infection and damage caused by micro-organisms. In healthy individuals, it's activation is followed by activation of the adaptive immune system. Various mechanisms are involved in innate immunity. These include mucociliary clearance (allowing physical removal of debris and inhaled microorganisms){Liote, 1989 306 /id}, clearance of pathogens by local inflammatory cells, secretion of cytokines, antimicrobial peptides and surfactant proteins by epithelial cells, local inflammatory cells and submucosal glands{Kaliner, 1992 309 /id;Ganz, 2004 308 /id}, and interaction with the adaptive immune system. Failure of innate and adaptive immune responses may result in microbial colonization and recurrent/persistent infections.

Clearance of fungi by local inflammatory cells: mainly eosinophils?

Normal cellular immune responses vary 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. Although eosinophils, neutrophils, macrophages and monocytes are all important antifungal effector cells, most research in CRS has focused on the role of eosinophils in antifungal immune defense.

Since fungi and eosinophils are co-localized in nearly all CRS tissue specimens, a cause-effect relationship between fungi and eosinophils has been suggested{Ponikau, 1999 20 /id;Ponikau, 2003 243 /id;Ponikau, 2005 241 /id}. Although Wei et al recently demonstrated a concentration-dependent increase in (CRS) eosinophil migration towards both CRS nasal mucin and CRS nasal tissue extracts{Wei, 2003 14 /id}, suggesting that fungi may trigger inflammatory cells to initiate a complex localized eosinophilic reaction, one should note that most CRS patients in this study were diagnosed with either asthma (9/10 patients) and/or atopy (4/10 patients). Since eosinophils from subjects with asthma (both allergic and non-allergic asthma) are known to exhibit a primed phenotype that is likely the consequence of eosinophil interaction with cytokines in the peripheral blood, resulting in increased eosinophil migration-, adhesion-, and degranulation capacity, it may well be that the presence of asthma and/or atopy explains the observed concentration-dependent increase in eosinophils migration{Griffin, 1991 275 /id;Koenderman, 1996 276 /id}, a hypothesis supported by recent data in sheep. In sheep, primed eosinophils were shown to be more effective in immobilizing and killing gastrointestinal parasites in the presence of specific anti-parasite antibodies in comparison to unprimed eosinophils{Rainbird, 1998 318 /id}.

Cytokines and chemokines: are they involved in antifungal immune defense?

Cytokines and chemokines are low molecular weight proteins with growth, differentiation, and activation functions that regulate and determine the nature of both innate and adaptive immune responses{Borish, 2003 22 /id}. Various cytokines and chemokines have been implicated in CRS pathogenesis{Fokkens, 2007 13 /id;Fokkens, 2007 16 /id}. Recently, striking differences were observed between CRS

and healthy control peripheral blood mononuclear cell (PBMC) cytokine responses when cultured with extracts from 4 common airborne fungi (*Alternaria, Aspergilllus, Cladosporium* and *Penicillium*). When cultured with *Alternaria* extract, PBMCs of CRS patients produced significantly more IL-5 and IFN-γ in comparison to healthy control PBMCs. In addition, some PBMCs of CRS patients produced more IL-5 in response to *Aspergillus* and *Cladosporium* extracts. PBMCs from all CRS patients produced IL-13 upon culture with all tested fungal extracts{Shin, 2004 242 /id}. Together, these data suggest that fungi may induce PBMC cytokine production (mainly T_H2 cytokines). Unfortunately, 61% of the patients demonstrated increased IgE levels to common aeroallergens and 78% suffered from bronchial asthma. Since PBMCs from asthmatic subjects (both allergic and non-allergic) are known to produce more IL-5 in response to allergen in comparison to both allergic rhinitic subjects and healthy controls{Haselden, 2001 277 /id} and since a second study (in which the frequency of atopy was equally distributed) showed only minimal changes in IL-5 and IFN-γ expression upon culture with *Aspergillus* and *Alternaria* extracts{Douglas, 2007 296 /id}, the role of fungi in inducing a T_H2 cytokine response remains to be determined.

Antimicrobial peptides: decreased levels may be involved in CRS pathogenesis

Immunocompetent hosts, when exposed to fungi, increase their production in cationic antimicrobial peptides to protect themselves against fungal invasion. Cathelicidins and defensins are two major families of cationic antimicrobial peptides involved in innate immunity at mucosal surfaces. Recently, Ooi et al demonstrated that LL-37, the free C-terminal peptide of human cathelicidin hCAP18 (human cationic antimicrobial peptide 18kDa), is significantly upregulated in a dose-response effect at

the mRNA and protein level in CRS patients without eosinophilic mucin in response to *Aspergillus fumigatus* and *Alternaria tenuis*. However, in CRS patients with eosinophilic mucin (but without fungal presence) no significant increase in LL-37 was observed at either the mRNA or the protein level in response to *Aspergillus* challenge. No increase in expression in both tissue and secreted LL-37 was observed upon *Alternaria* challenge{Ooi, 2007 297 /id}. Although the idea is interesting, since neither CRS patients with eosinophilic mucin and fungal presence nor a control group were included in this study, the exact role of LL-37 in the CRS pathogenesis remains to be determined.

Besides cathelicidins and defensins, various other antimicrobial peptides, including lactoferrin, lysozyme and secretory leukoprotease inhibitor, have been identified in nasal secretions{Cole, 2002 33 /id} and sinus mucosa{Fukami, 1993 310 /id}. Lactoferrin possesses a variety of functions, including antibacterial, antifungal, and antiviral activities{Cavestro, 2002 34 /id}. More recently, this peptide has been shown to possess antibiofilm properties{Singh, 2002 128 /id}. Bacterial biofilms are present in the majority of CRS patients and may contain large amounts of fungal elements{Healy, 2008 314 /id}. Downregulation of lactoferrin was recently observed in those CRS patients with nasal polyps{Psaltis, 2007 313 /id} and/or those with biofilms{Psaltis, 2008 312 /id}. However, no difference was observed between CRS patients with or without eosinophilic mucin, those with or without fungal allergy and those with or without fungi present{Psaltis, 2007 313 /id;Psaltis, 2008 312 /id}.

Surfactant proteins: absence of surfactant protein D may result in failure to clear fungi from the nose and paranasal sinuses

Pulmonary surfactant is a mixture of phospholipids and proteins. Four different surfactant proteins (SPs) are known to exist: SP-A, SP-B, SP-C and SP-D{Crouch, 2000 104 /id}. SP-D binds and agglutinates micro-organisms and enhances phagocytosis, chemotaxis, and cytokine production. SP-D has been shown to play an important role in the immune response to *Aspergillus fumigatus* in the lung and is present in submucosal glands of CRS patients without eosinophilic mucin, CRS patients with eosinophilic mucin but without fungal allergy and healthy controls. Highest levels are detected in healthy controls. In CRS patients with eosinophilic mucin and fungal allergy, however, SP-D protein remains below detection levels. *In vitro* studies demonstrate that *Alternaria tenuis* upregulates SP-D mRNA in those CRS patients with and those without eosinophilic mucin. *Aspergillus fumigatus*, on the other hand, increases SP-D mRNA expression in CRS patients without eosinophilic mucin only{Ooi, 2007 298 /id}. Absence of SP-D protein may result in failure to clear fungi from the nose and paranasal sinuses and, as a result, disease development.

FUNGUS ANTI-HOST EFFECTS

Besides innate and adaptive antifungal immune responses that may contribute to disease development, fungus anti-host effects may be involved in CRS pathogenesis. Ubiquitous airborne fungi (especially *Alternaria* and *Aspergillus*) are known to 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 and release of mediators involved in tissue damage{Vroling,

2008 317 /id;Kauffman, 2000 283 /id;Reed, 2004 284 /id}. In addition to an indirect effect, recent studies indicate that *Alternaria alternata* may activate eosinophils directly. *Alternaria alternata,* but not IL-5, has been shown to induce eosinophil IL-8 synthesis and eosinophil surface expression of CD11b (a β_2-integrin that is used by eosinophils to adhere to to β-glucan, a major fungal cell wall component{Yoon, 2008 315 /id}) and CD63 (a component of eosinophil granule membranes) in healthy volunteers, patients with allergic rhinitis and patients with bronchial asthma. Upon recognition of *Alternaria alternata*, eosinophils released eosinophil derived neurotoxin (EDN){Inoue, 2005 239 /id} and this response may play a pivotal role in CRS pathogenesis.

THERAPY

Topical and oral antifungals: ineffective in the treatment of CRS patients

If CRS inflammation is caused by an immune reaction to fungi, reducing the presence of this inflammatory trigger may improve the course of the disease{Ponikau, 1999 20 /id}. 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 and amphotericin B were shown to be most effective, independent of fungal organism tested{Bent, 1996 249 /id}. Despite the clinical effectiveness, the use of systemic antifungals is limited by adverse systemic reactions. Topical treatment may have the advantage 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{Kfoury, 1997 93 /id} and bladder{Leu, 1995 234 /id}. Recently, amphotericin B nasal lavages have been advocated in the treatment of CRS (table 1). Although safe to use and despite initial evidence of benefit of topical amphotericin B irrigations in two uncontrolled trials{Ponikau, 2002 244 /id;Ricchetti, 2002 80 /id}, one subsequent uncontrolled prospective trial{Helbling, 2006 316 /id} and four subsequent double-blind placebo-controlled studies investigating the effect of topical amphotericin B irrigations{Ponikau, 2005 8 /id;Weschta, 2004 5 /id;Ebbens, 2006 299 /id;Liang, 2008 117 /id} and nasal spray{Weschta, 2004 5 /id;Helbling, 2006 316 /id} either failed to show benefit{Weschta, 2004 5 /id;Helbling, 2006 316 /id;Ebbens, 2006 299 /id;Liang, 2008 117 /id} or showed, at best, only modest radiological benefit without symptomatic improvement{Ponikau, 2005 8 /id} in CRS patients with or without nasal polyps. Since Weschta et al demonstrated that fungal eradication did not alleviate symptoms, it should be questioned whether fungal eradication is involved in disease resolution{Weschta, 2004 5 /id}. Whether dosage, treatment time and route of administration have an impact on treatment outcomes, requires additional research. This is especially true since recent *in vitro* data suggest that amphotericin B nasal lavages are ineffective in killing fungi at concentrations of 100 µg/mL (dosages used by both Ponikau et al{Ponikau, 2002 244 /id} and Ebbens et al{Ebbens, 2006 299 /id}) when used for 6 consecutive weeks{Shirazi, 2007 26 /id}. Irrigations with concentrations of 200 µg/mL and 300 µg/mL were shown to successfully prevent fungal growth at 5 and 6 weeks respectively{Shirazi, 2007 26 /id}. Whether prolonged treatment with topical amphotericin B at a concentrations of 100 µg/mL is equal to

treatment with concentrations of 200 and 300 µg/mL for shorter periods of time remains, to date, unclear.

Although several uncontrolled reports have suggested that oral antifungal agents are effective in the treatment of CRS{Rains, 2003 287 /id}, in a recent double-blind placebo-controlled study Kennedy et al treated 53 CRS patients with high-dose oral terbinafine (625 mg/day) for a period of 6 weeks and demonstrated no improvement in subjective and objective outcome measures{Kennedy, 2005 81 /id}, results in line with previous results on topical antifungal treatment{Weschta, 2004 5 /id;Ebbens, 2006 299 /id;Liang, 2008 117 /id}. Thus, the use of both topical and oral antifungals in the treatment of patients with CRS is not substantiated by the majority of publications.

Topical amphotericin B: if it does work, how does it work?

Although the use of topical amphotericin B in the treatment of CRS is not substantiated by the majority of publications, dosage and duration of treatment may have influenced outcome. If we assume that topical amphotericin B is effective in the treatment of CRS patients, various mechanisms may be involved. First and most likely, amphotericin B may reduce fungal load and, as a consequence, the inflammatory response in the nose and paranasal sinuses. Second, amphotericin B may have a direct (cytotoxic) effect on nasal polyp epithelial cells. Although amphotericin B is a sterol-binding agent with high affinity for ergosterol (the dominant fungal sterol) and low affinity for cholesterol (the mammalian sterol), recent evidence suggests that topical amphotericin B is able to modify the cell membrane structure of nasal polyp epithelium but not of inferior turbinate epithelium by forming aqueous pores, resulting in increased membrane permeability and, as a consequence,

disruption of nasal polyp epithelial cells{Jornot, 2003 290 /id}. The underlying mechanism remains to date unclear. Third, amphotericin B may have a direct anti-inflammatory effect on CRS mucosa. Although interesting, to date no data exist confirming this hypothesis and the three studies published in this field report no difference in cytokine, chemokine and growth factor responses between placebo and amphotericin B{Shin, 2004 292 /id}{Weschta, 2006 259 /id}{Ebbens, 2008 347 /id}.

Immunotherapy: effective in those CRS patients with concurrent fungal allergy

If CRS stems from a hypersensitivity to retained fungal elements (a conclusion that should be questioned as mentioned previously) the removal of fungal elements may minimize ongoing stimulation. However, when the underlying hypersensitivity remains untreated, the disease is expected to recur. From 1994 onwards, Mabry et al prospectively treated 23 patients with AFS with antifungal immunotherapy following thorough exenteration of the involved sinuses. A decreased need for both systemic and topical corticosteroids, a marked decrease in polyp recurrence and a lessening of long-term nasal and sinus crusting was observed over a treatment period of 1-3 years in 11 patients{Mabry, 1998 300 /id}. Cessation of immunotherapy after 3 years did not result in recurrence of symptoms in the 7 to17 months follow-up{Mabry, 2000 301 /id}. When interpreting these data, one should note that no placebo group was included, that controls included those patients that dropped out from the immunotherapy group, that several patients were lost to follow-up, that most patients were treated with immunotherapy to both fungal and non-fungal antigens and that all patients were treated with nasal irrigations and topical steroids for a variable period of time post-operatively. But, even though many confounders are present, the results of this study are intriguing. Even if one assumes that fungal allergy is not causative of

CRS, one may conclude that antifungal immunotherapy is effective in reducing signs and symptoms in those CRS patients with concurrent fungal allergy. Future placebo-controlled studies are necessary to reveal the true role of antifungal immunotherapy in the treatment of CRS patients.

Conclusions and future directions

The role of fungi in CRS remains to be defined. Although different studies have agreed that fungi can be detected in the nose and paranasal sinuses of nearly all CRS patients, they 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 there are many mechanisms by which fungi can exert an effect on sinus mucosa in susceptible individuals. Future studies will have to clarify the role of fungi in CRS, which fungal organisms, if at all, may be pathogenic, and what exactly characterizes the immunologic response to fungi that may potentially results in the development of disease. 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.