

Allergenic fungi in allergic fungal sinusitis

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Background: Patients with allergic fungal sinusitis demonstrate skin test reactivity to many fungal extracts. Various fungi have been isolated from the characteristic allergic mucin.

Objective: This study was designed to identify allergens in allergic mucin and to compare them to those found in commercial fungal extracts.

Methods: Allergic mucin was collected during functional endoscopic sinus surgery from 11 patients meeting strict diagnostic criteria for allergic fungal sinusitis and from three allergic rhinitis patients with chronic sinusitis. A portion was solubilized in saline and centrifuged. To identify allergens, proteins in allergic mucin and fungal extracts were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and immunostained using patient sera and enzyme-labeled anti-human IgE.

Results: All patient sera recognized numerous bands ranging from 18 to 90 kD. In mucin, bands were consistently found in the 35 to 50 kD range. Corresponding bands in fungal extracts were found in only 1/11 patients with allergic fungal sinusitis. Sera from 4/11 patients detected an 18-kD protein in allergic mucin, but sera from all patients with allergic fungal sinusitis recognized an 18-kD protein in commercial fungal extracts. Sera from selected patients with allergic fungal sinusitis detected human epithelial proteins in the 35 to 50 kD range.

Conclusions: Fungal allergens were not detected in allergic mucin of all patients with allergic fungal sinusitis. The 18-kD allergen appears to be shared by many fungi, and may be a fungal panallergen. The source of the apparent allergens in the 35-50-kD range warrants further study.

Ann Allergy Asthma Immunol 1997;79:431-5.

INTRODUCTION

In 1981 Millar described five patients with fungal sinusitis who had clinical features similar to allergic bronchopulmonary mycosis.¹ Two years later Katzenstein described the histopathology of allergic mucin and named the entity allergic aspergillus sinusitis.² Allergic fungal sinusitis, once considered rare, has been recognized more frequently. deShazo has reviewed all reported cases of allergic fungal sinusitis and found that the entity is widely distrib-

uted in the United States; large case series have been reported from Arizona, Missouri, Texas, and Alabama.³ The majority of fungi isolated belong to the non-*aspergillus* dematiaceous group.⁴⁻⁸ Allergic fungal sinusitis appears to be a hypersensitivity response to fungi in the paranasal sinuses of atopic, immunocompetent individuals characterized by a typical "allergic mucin." Allergic mucin is usually greenish-brown with a peanut butter-like consistency, prominent eosinophilia, Charcot-Leyden crystals, sloughed respiratory epithelium, and fungal hyphae without tissue invasion. Patients typically demonstrate skin prick test reactivity to many different commercial fungal extracts, rather than to just the major organism isolated from allergic mucin.

This study was designed to determine whether fungal allergens could

be identified in allergic mucin of patients with an unequivocal diagnosis of allergic fungal sinusitis and whether sera from patients with allergic fungal sinusitis might recognize allergens in various commercial fungal extracts. Sera from 11 patients with a firm diagnosis of allergic fungal sinusitis detected an 18-kD band by IgE immunoblotting in 4/11 mucin samples and in all commercial extracts tested. Sera from 10/11 patients also recognized 35 to 50 bands in mucin that were not present in commercial fungal extracts. Since the allergic mucin contains sloughed respiratory epithelium in addition to fungal elements, immunoblotting was used to compare mucin proteins with those of an epithelial extract in three arbitrarily selected patients with allergic fungal sinusitis. The 30 to 50-kD bands detected in allergic mucin were recognized in the epithelial extract in two of three patients with allergic fungal sinusitis.

MATERIALS AND METHODS

The allergy-immunology and otolaryngology services prospectively evaluated 35 patients for possible allergic fungal sinusitis as part of a clinical investigation protocol approved by the human assurance committee. Patients received modified prick skin testing with a bifurcated needle (Allergy Laboratories of Ohio, Columbus, OH) to 33 fungal allergen extracts in addition to histamine (1 mg base/mL), compound 48/80 and glycerosaline controls. All extracts were purchased from a commercial vendor (MMP, Bayer, Spokane, WA), except for *Bipolaris spicifera* which is not commercially available. Source material for *B. spicifera* was obtained from the American Type Culture Collection as *Cochliobolus spicifer* (the perfect state of *B. spicifera*); the organism was grown in bulk quantity in Czapek-Dox broth and a

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Financial support: Thinhouse Allergy Research Foundation, Medical College of Georgia.

Received for publication November 25, 1996.

Accepted for publication in revised form May 19, 1997.

mycelial extract for epicutaneous skin testing was prepared as previously described.⁸ Briefly, the fungal mat was defatted in acetone, dried, ground with a mortar and pestle, extracted at 1:20 wt/vol overnight at 4 °C in ammonium bicarbonate buffer, pH 8.0, centrifuged to remove particulate matter, and filtered with a 0.45- μ m syringe filter (Corning, Corning, NY). Skin prick test results were considered positive if the wheal was at least 3 mm greater than that produced by the negative control.

Patients meeting five of the six following criteria were given an unequivocal diagnosis of allergic fungal sinusitis: (1) history and physical examination not suggesting another etiology; (2) sinus CT consistent with allergic fungal sinusitis; (3) typical allergic mucin with fungal hyphae, eosinophils and Charcot-Leyden crystals obtained at the time of surgery; (4) positive fungal culture of allergic mucin; (5) presence of specific IgE, either by epicutaneous skin testing or in vitro assays;

and (6) elevated total serum IgE (>150 IU/mL).

A portion of allergic mucin collected at the time of functional endoscopic surgery was sent to an expert mycology laboratory (Dr. Sid Crow, Georgia State University, Atlanta) for culture and identification. A 0.5-gram portion was solubilized in 2-mL sterile saline and centrifuged; the supernatant was collected and stored at -70 °C. A 1-mL volume of the mucin supernatant was concentrated by lyophilization with reconstitution in 100 μ L distilled

Table 1. Skin Prick Test Reactivity and Fungal Culture Results* in 11 Patients with Definite Allergic Fungal Sinusitis

Fungal Extract	Patients										
	2	3	4	5	8	13	14	16	17	22	25*
<i>Alternaria alternata</i>	⊕	+	-	+	⊖	-	+	⊕	+	-	nd
<i>Cladosporium cladosporioides</i>	+	⊖†	+	+	-	-	+	-	+	-	nd
<i>Curvularia spicifera</i>	+	+	+	+	-	-	+	+	⊕§	-	⊕
<i>Helminthosporium interseminatum</i>	+	+	+	+	-	+	+	+	-	-	nd
<i>Stemphylium botryosum</i>	+	+	+	-	-	-	+	+	+	-	nd
<i>Pullularia pullulans</i>	+	+	+	-	-	-	+	-	+	-	nd
<i>Aspergillus mix</i>	+	+	+	-	+	-	-	+	+	-	nd
<i>Penicillium mix</i>	+	+	⊕‡	+	-	-	-	-	-	-	nd
<i>Monilia sitophila</i>	+	-	+	-	-	-	-	-	+	-	nd
<i>Gliocladium fimbriatum</i>	+	-	+	+	-	-	-	-	-	-	nd
<i>Trichoderma viride</i>	-	-	-	-	-	-	+	-	+	-	nd
<i>Cephalosporium acremonium</i>	+	+	+	-	-	-	+	-	+	-	nd
<i>Paecilomyces varioti</i>	+	-	-	-	-	-	+	+	-	-	nd
<i>Epicoccum nigrum</i>	+	-	-	-	-	-	+	-	+	+	nd
<i>Fusarium vasinfectum</i>	-	-	-	+	-	-	⊕	+	+	+	nd
<i>Rhodotorula glutinis</i>	-	+	+	+	-	-	+	-	-	+	nd
<i>Phoma herbarum</i>	+	+	+	-	-	-	-	+	-	-	nd
<i>Chaetomium globosum</i>	+	-	-	-	-	-	-	+	+	-	nd
<i>Saccharomyces cerevisiae</i>	-	+	-	-	-	-	-	-	-	-	nd
<i>Pleospora sp.</i>	+	+	-	+	-	-	+	+	+	+	nd
<i>Mucor racemosus</i>	-	-	-	-	-	-	-	-	-	+	nd
<i>Rhizopus nigricans</i>	-	-	-	-	-	-	-	-	-	-	nd
<i>Botrytis scinerea</i>	+	-	-	-	-	-	+	+	+	+	+
<i>Nigrospora sphaerica</i>	+	+	-	-	-	-	+	-	+	+	nd
<i>Microsporium mix</i>	+	-	+	-	-	-	+	+	-	+	nd
<i>Trichophyton mix</i>	-	-	+	-	-	-	-	+	-	+	nd
<i>Candida albicans</i>	-	-	-	-	-	-	-	-	-	-	nd
<i>Mycogone sp.</i>	-	+	-	-	-	-	+	-	-	-	nd
<i>Scopulariopsis brevicaulis</i>	+	-	-	-	-	-	-	-	-	-	nd
<i>Geotrichum candidum</i>	+	-	-	-	-	-	-	-	-	-	nd
Corn smut	-	-	-	-	-	-	+	-	-	-	nd
Oat smut	-	-	-	-	-	-	-	-	-	-	nd
<i>Bipolaris spicifera</i>	nd	nd	nd	Ⓝ	nd	Ⓝ	nd	nd	nd	Ⓝ	nd
Total positive skin prick tests	21	14	14	9	1	1	18	13	15	9	

* Organisms cultured from allergic mucin are circled.

† Culture positive for *Cladosporium herbarum*.

‡ Culture positive for *Penicillium funiculosum*.

§ Culture positive for *Curvularia lunata*.

|| Skin testing not done in this patient with positive in vitro test to *Curvularia lunata* and *Botrytis scinerea*.

nd = not done.

water. Additionally, sinus contents were obtained from three patients with allergic rhinitis at the time of functional endoscopic sinus surgery required for chronic sinusitis. These samples were treated in the same fashion as the allergic mucin.

Undiluted and concentrated allergic mucin supernatants obtained from 11 patients meeting the above criteria were separated by molecular weight using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli.⁹ For each patient, three fungal extracts were separated by SDS-PAGE along with the allergic mucin. One fungal extract was selected to match the organism (genus and species) isolated from the patients' mucin culture as closely as possible. The other two fungal extracts, *Cladosporium cladosporioides* and *Cephalosporium acremonium*, were chosen arbitrarily. To determine which allergic mucin and fungal proteins were recognized by patient's IgE, each gel was placed between two sheets of nitrocellulose and allowed to transfer overnight. The next day, the nitrocellulose containing the transferred proteins was first incubated with patient serum, washed, and immunostained with goat alkaline phosphatase-labeled anti-human IgE (Sigma, St. Louis, MO).

A human epithelial extract was prepared as described by Ochs.¹⁰ Briefly, 4 mg of skin epithelial shavings was collected and extracted in SDS sample buffer for 20 minutes. After extraction, the sample was centrifuged and frozen at -70°C for future analysis by the same procedure as the allergic mucin and fungal extracts.

RESULTS

A majority (7/11) of the eleven patients who met the criteria for diagnosis of unequivocal allergic fungal sinusitis demonstrated skin test reactivity to a broad range of commercial fungal extracts. Fungal cultures from allergic mucin matched positive skin test results in 6/11 patients with allergic fungal sinusitis, either to genus or genus and species of the commercial fungal extracts. Skin testing to *Bipo-*

laris spicifera was not performed in these eleven patients due to unavailability of the extract at the time the patients were evaluated.

In Western immunoblotting, sera from patients with allergic fungal sinusitis recognized proteins in the 18 to 90-kD range in autologous allergic mucin obtained at surgery. Sera from all 11 patients with allergic fungal sinusitis recognized an 18-kD protein present in the commercial extracts as demonstrated in lanes 2 and 3 of Figure 1. Additionally, sera from 4 of 11 patients with allergic fungal sinusitis identified an 18-kD protein in the separated mucin. This 18-kD band corresponded well to the 18-kD protein found in the commercial fungal extracts (Fig 2). Additional bands were not found when concentrated mucin samples were analyzed. Sera from 10 of 11 patients consistently recognized a group of proteins at 35 to 50-kD in allergic mucin (Figs 1 and 2). Only one

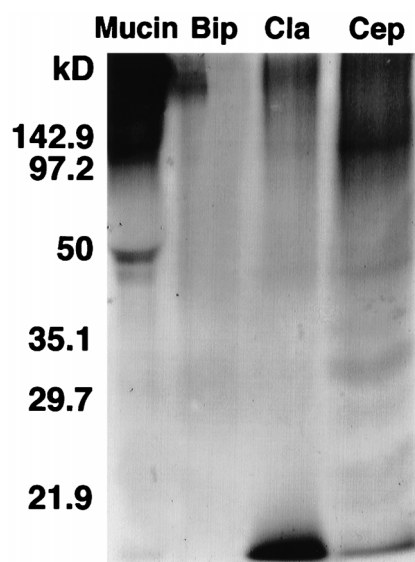


Figure 1. Immunoblot comparing allergic mucin from allergic fungal sinusitis patient numbers 5 to 3 commercial extracts. Lane 1: Allergic mucin, culture positive for *Bipolaris* species, with discrete bands at 44 and 50 kD. Lane 2: *Bipolaris spicifera* extract. Lane 3: *Cladosporium cladosporioides* extract. Lane 4: *Cephalosporium acremonium* extract. Lanes 3 and 4 contain a weak 44-kD band and a prominent 18-kD band.

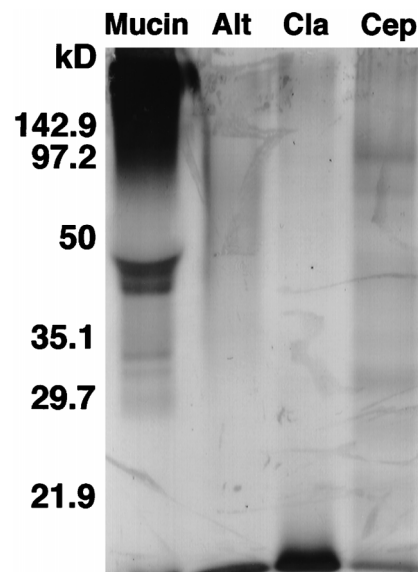


Figure 2. Immunoblot of allergic mucin from allergic fungal sinusitis patient numbers 2 and 3 commercial extracts. Lane 1: Allergic mucin, culture positive for *Alternaria alternata*, with distinct bands between 31 to 43 kD. Lane 2: *Alternaria alternata* extract. Lane 3: *Cladosporium cladosporioides* extract. Lane 4: *Cephalosporium acremonium* extract. No comparable bands are present in the 31 to 43-kD range. An 18-kD band is present in allergic mucin and the three fungal extracts tested.

patient with allergic fungal sinusitis demonstrated protein bands in both mucin and the commercial *Cephalosporium acremonium* (Fig 1, lanes 1 and 4). Sera from two of three arbitrarily selected patients with allergic fungal sinusitis identified 35 to 50-kD bands in separated allergic mucin and the human epithelial extract (Fig 3).

Sera from all three allergic rhinitis patients with chronic sinusitis also recognized 35 to 50-kD proteins in sinus contents obtained at surgery. In addition, sera from the same patients recognized a similar 18-kD allergen present in some of the commercial fungal extracts; however, this 18-kD band was not identified in their separated sinus contents (Fig 4).

DISCUSSION

Allergic fungal sinusitis is considered to be a type I hypersensitivity response to fungi, with both clinical and histo-

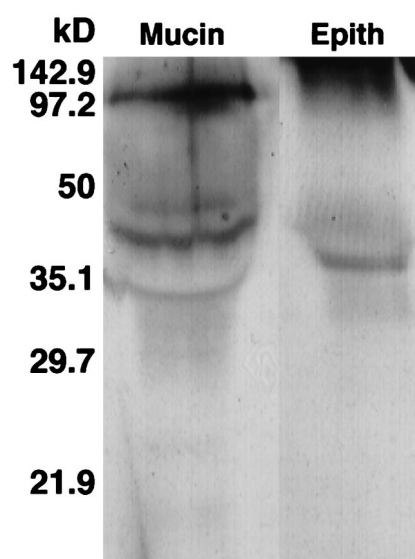


Figure 3. Immunoblot comparing allergic mucin from allergic fungal sinusitis patient 2 with the epithelial extract. *Lane 1*: Allergic mucin with prominent bands between 35 to 42 kD. *Lane 2*: Epithelial extract with a band at 38 kD.

logic similarities to allergic bronchopulmonary mycosis. When fungal cultures of allergic mucin are performed, the majority of fungi isolated belong to the dematiaceous group. Unlike *Aspergillus*, the dematiaceous fungi, which contain melanin in their cell walls, tend to cause unilateral sinus disease, sinus expansion, and bony erosion. They are more likely to produce complications like proptosis; representative genera of dematiaceous fungi are *Bipolaris*, *Curvularia*, *Exserohilum*, *Alternaria*, and *Cladosporium*.¹¹ Manning et al identified dematiaceous fungi in mucin from 16 of 24 patients with allergic fungal sinusitis; *Bipolaris* species were cultured from 11 patients and *Curvularia* species were encountered in four patients.¹² In the present study, non-*aspergillus* fungi (mostly in the dematiaceous group) were most prevalent in allergic mucin cultures. *Alternaria* and *Bipolaris* were isolated in three patients each, *Curvularia* in two patients, and *Cladosporium* in one patient.

Western blot analysis demonstrated that sera from patients with allergic fungal sinusitis recognize a wide range

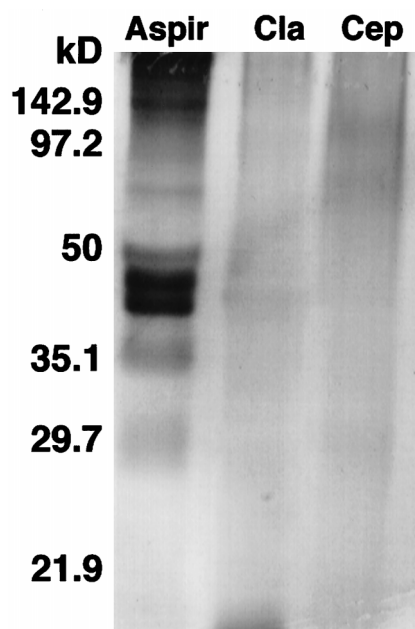


Figure 4. Immunoblot from patient with allergic rhinitis and bacterial sinusitis compared with commercial fungal extracts. *Lane 1*: Bacterial sinus aspirate with prominent bands between 35 to 50 kD. *Lane 2*: *Cladosporium cladosporioides* extract. *Lane 3*: *Cephalosporium acremonium* extract. Lanes 2 and 3 do not identify bands between 35 to 50 kD. An 18-kD band is present in lanes 2 and 3, not present in lane 1.

of proteins, from 18 to 90 kD, in allergic mucin and commercial fungal extracts. Sera from patients with allergic fungal sinusitis recognized an 18-kD protein present in all commercial fungal extracts examined. In 4 of 11 patients with allergic fungal sinusitis, sera also recognized an 18-kD protein in autologous allergic mucin. Sera from the three patients with chronic sinusitis also identified an 18-kD allergen in fungal extracts but not in autologous sinus contents. The identity of the 18-kD protein(s) seen in allergic mucin and fungal extracts is unknown, but its low molecular weight and ubiquitous nature suggests that it might be a fungal panallergen, perhaps even a profilin.¹³ This finding would explain the multiple fungal skin test reactivity found in this study; clearly, further characterization of this protein is warranted. A review of the literature reveals no previous studies in which al-

lergic mucin has been examined for the presence of fungal allergens. The finding of skin test reactivity to multiple fungal extracts has previously been reported.^{5,8}

Sera from 10 of 11 patients with allergic fungal sinusitis consistently recognized 35 to 50-kD proteins in allergic mucin, corresponding only weakly with commercial fungal extract proteins of a similar molecular weight in just one patient. It is likely that allergens produced by fungi growing in allergic mucin at body temperature could be different from those produced in the culture conditions used for the manufacture of allergen extracts. Species or strain differences might also explain this discrepancy; producing extracts of fungi actually isolated from allergic mucin was not possible in this study. The mucin solubilization procedure may not have adequately extracted fungal allergens. Perhaps fungal allergens were present in allergic mucin at concentrations too low for detection, even after concentration of the allergic mucin supernatant. Additional limitations of fungal allergen extracts include lack of standardization, potential extract batch and species variation, and variable expression of allergens even when culture techniques are consistent. Nonetheless, all patients had demonstrated skin test reactivity to the extracts employed in this study.

Since all patient sera clearly identified bands in the 35 to 50-kD range, it is important to identify the recognized protein or proteins. Unfortunately, only a limited number of fungal allergens have been isolated and characterized.^{14,15} Allergic mucin also contains mucosal epithelial cellular debris, as would the material obtained from the chronic sinusitis patient at the time of sinus surgery. The finding that sera recognize proteins in this molecular weight range in a skin epithelial extract does not prove that these proteins share identical or similar epitopes, but raises the question of whether a human antigen, an "autoallergen," is in some way involved in the pathogenesis of allergic fungal sinusitis or chronic sinusitis in allergic rhinitis patients. If so, it would

be easier to understand why medical management of patients with allergic fungal sinusitis after extensive surgical debridement of the sinuses is so difficult, consistently requiring chronic oral corticosteroid therapy. More sophisticated analytical techniques will be required to determine whether fungi are directly responsible for chronic course of allergic fungal sinusitis, are initiating factors only, or are merely innocent bystanders.

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