Prevalence of allergic sensitization to indoor fungi in West Virginia

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ABSTRACT

Exposure to indoor fungi is of growing concern in residential and occupational environments in the United States. The purpose of this study was to determine the prevalence of sensitization to common indoor fungal species in an atopic population. We evaluated 102 patients (73 female and 29 male patients) for immunoglobulin E (IgE) reactivity to a panel of skin-prick test (SPT) reagents used for routine allergy testing. Patients also were tested for six additional fungi that are common indoor contaminants. All patients had symptoms consistent with allergic rhinitis or asthma. The presence of specific IgE against the fungal species was determined using immunoblotting. Of the 102 eligible patients, 68% had at least one positive skin test. The most prevalent positive SPTs were to dust mites, cats, vernal grass, and short ragweed. Overall, 21/102 (21%) patients with asthma or allergic rhinitis were skin test positive to at least one fungal extract. Of the patients with a positive SPT to fungi, 12/21 (58%) showed sensitivity to one or more of the newly tested species; most notably Trichoderma viride *(8%),* Chaetomium globosum *(7%),* Paecilomyces variotii *(7%), and* Acremonium strictum *(6%). Immunoblotting revealed specific IgE against a number of protein bands belonging to these fungal species. The prevalence of fungal sensitization was common, particularly for indoor fungal contaminants that are not routinely included in SPT panels. Cross-reactivity with other fungi may partially explain our results; however, skin testing for these indoor fungi may provide useful diagnostic information.*

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Personal exposure to indoor fungi is of growing concern in a number of damp home and occupational environments in the United States.[1] Although indoor fungal exposure is associated with allergic reactions, only a small proportion of species have been studied in any detail. The involvement of fungi in causing adverse health effects remains unclear and exposure–response relationships for adverse health effects is generally unknown.[2] The lack of such information is primarily caused by inadequacies of current monitoring techniques.[3] The recent development of new immunodetection techniques has provided unique insight into the factors influencing airborne fungal exposures. These techniques have shown that previously unrecognized species as well as fungal fragments have a significant contribution to the aeroallergen load.[4,5] However, the association between these newly recognized fungal components and respiratory disease remains unknown and direct evidence as to their involvement is lacking.

Indoor fungi are highly diverse between geographic locales throughout the United States.[6] A number of studies have identified specific fungal genera, in particular, *Trichoderma*, *Chaetomium*, *Paecilomyces*, and *Acremonium*, to be common in many of these environments[6]; however, very little is known about their impact on exacerbating health effects. Recent studies suggest that these fungi may be important sensitizing sources and infectious disease agents.[7] To better understand etiologic disease relationships between specific fungal exposure and clinical symptoms, accurate information on the distribution of fungi in the ambient environment of the patient and the prevalence of sensitization to these species is required. The aim of this study was to determine the prevalence of sensitization to common

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indoor fungal species in a sample of the West Virginian population.

MATERIALS AND METHODS

Human Subjects

Prevalence of immunoglobulin E (IgE) to various fungi was determined by skin-prick test (SPT) in the West Virginia University Pediatric Allergy Clinic. We recruited a total of 112 participants from a suburban/rural area who were undergoing SPTs to common allergens for evaluation of allergic rhinitis or asthma. Inclusion criteria were that the subject was ≥8 years of age and able to personally provide informed consent for participation in the study (or in the case of a child, the child's parent or legal guardian). Exclusion criteria included the inability to obtain informed consent or a negative histamine test. Ten subjects were removed because they did not meet the criteria and two subjects were not tested with the additional indoor fungal extracts. Subjects with one or more positive SPTs to fungi were asked to provide a blood sample. Blood samples were then transferred to the National Institute for Occupational Safety and Health (NIOSH) and stored at −20°C until used for Western blot analysis. This study was approved by the Institutional Review Board (IRB 16047) of West Virginia University and the NIOSH Human Subject Review Board, and informed consent was obtained from all participants.

Reagents

The panel of SPT reagents routinely used for allergy testing comprised environmental, insect, grass, weed, tree, and fungal extracts including *Alternaria alternata*, *Aspergillus fumigatus*, *Cladosporium sphaerospermum* (*Hormodendrum hordei*), *Penicillium notatum, Dreschlera sorokiniana* (*Helminthosporium sativum*), *Epicoccum nigrum* (syn. *Epicoccum purpurascens*), *Pullaria* spp., and *Rhizopus* spp. In addition, we evaluated eight additional extracts from six fungi commonly found indoors including *Stachybotrys chartarum* (Antigen Laboratories, Liberty, MO), *Trichoderma viride* (Antigen Laboratories and Greer Laboratories, Lenoir, NC), *Chaetomium globosum* (Antigen Laboratories; Greer Laboratories), *Acremonium strictum* (Antigen Laboratories), *Paecilomyces variotii* (Greer Laboratories), and *Penicillium chrysogenum* (Greer Laboratories). These fungi were chosen because they frequently are found as indoor contaminants in the northeastern United States (R.S. Pore, WV University, personal communication, 2004).

Skin Testing

SPTs were performed by placing a drop of test solution on the skin and pricking the epidermis beneath the drop with a disposable plastic lancet (DermaPIK II; Biomedex, Spokane, WA). SPT sites were wiped clean after 15 minutes and the wheal and flare reactions were carefully measured. Histamine (10 mg/mL) was used as a positive control and diluent (human serum albumin in 50% glycerol) as a negative control. Positive responses were defined as 4 mm or greater than that of the negative control and at least one-half the size of the histamine control. Three nonatopic control individuals tested negative to the eight additional extracts without any evidence of irritant reactions.

Fungal Isolates

Fungal isolates of *A. strictum* (ATCC 46646), *A. fumigatus* (NIOSH 173037), *A. alternata* (ATCC 11612), *C. globosum* (NIOSH 324001), *P. variotii* (ATCC 66705), and *T. viride* (ATCC 16640) were purchased from the American Type Culture Collection (Manassas, VA) or acquired from the NIOSH fungal reference collection. Isolates of each species were subcultured from stock sources and grown for 10 days at room temperature (RT) on malt extract agar (2% dextrose, 0.1% peptone, 2% malt extract, 2% agar; Difco, Becton Dickenson, Sparks, MD) until sporulation occurred. Spores were harvested and a suspension containing 1×10^7 spores/mL in PBS was used to inoculate a 250-mL Erlenmeyer flask containing 50 mL of liquid malt extract broth growth media (2% dextrose, 0.1% peptone, 2% malt extract; Difco, Becton Dickenson). Flasks were incubated at RT and rotated at 200 rpm. After 10 days, the hyphal-broth slurry was transferred to a 50-mL polypropylene falcon tube and centrifuged for 10 minutes at 5500 rpm. The hyphal pellet was washed with 5 mL of sterile ice-cold PBS to remove any remaining malt extract broth and then centrifuged for 10 minutes at 5500 rpm. The hyphal pellet was lyophilized before extraction.

Extract Preparation and Protein Determination

Lyophilized hyphal preparations were macerated using a mortar and pestle in liquid nitrogen. The powdered hyphal preparations were then extracted in 125 mM of NH_4HCO_3 buffer supplemented with 1 mM of phenylmethylsulfonylfluoride and 5 mM of EDTA at 10% w/v for 3 hours at 4°C under constant agitation using a rotary shaker. After extraction, particulate matter was removed by centrifugation for 10 minutes at 5500 rpm. The extracts were then lyophilized and resuspended in 1 mL of dH_2O and protein concentrations were determined according to the BCA method (Pierce Chemical Co., Rockford, IL).

Western Blot Analysis

Hyphal extracts of *A. strictum*, *C. globosum*, *P. variotii*, and *T. viride* were separated on 12% Tris-HCl gels (Ready Gel; BioRad, Hercules, CA) under denaturing conditions for 2 hours at 100 V. Prestained broad-range

Table 1 **SPT reactivity to a panel of common aeroallergens and indoor fungi**

Allergen	Number Positive*	Number Negative	Percent Positive	Allergen	Number Positive*	Number Negative	Percent Positive
Perennials				Trees			
Dust mite	40	62	39.2	Box elder	19	83	18.6
Cat	27	75	26.4	Ash	14	88	13.7
Cockroach	22	80	21.5	Elm	14	88	13.7
Dog	7	95	6.8	Birch	12	90	11.7
Mouse	4	98	3.9	Hickory	12	90	11.7
Insects				Oak	12	90	11.7
Caddis fly	16	86	15.6	Sycamore	8	94	7.8
House fly	16	86	15.6	Walnut	8	94	7.8
May fly	14	88	13.7	Beech	6	96	5.8
Moth	10	92	9.8	Juniper	5	97	4.9
Ant	6	96	5.8	Fungi			
Grasses				*A. alternata*	12	90	11.8
Vernal	27	75	26.4	*T. viride*#	8	92	8.0
Redtop	26	76	25.4	*C. globosum*#	7	93	7.0
Timothy	26	76	25.4	*P. variotti*	7	93	7.0
Fescue	25	77	24.5	*A. fumigatus*	7	95	6.9
Kentucky Bluegrass	23	79	22.5	*C. sphaerospermum*	7	95	6.9
Bermuda	20	82	19.6	*P. notatum*	7	95	6.9
Weeds				*A. strictum*	6	94	6.0
Short ragweed	27	75	26.4	*D. sorokiniana*	6	96	5.9
Giant ragweed	24	78	23.5	*E. nigrum*	5	97	4.9
Plantain	20	82	19.6	*C. globosum*§	4	96	4.0
Sour dock	18	84	17.6	*P. chrysogenum*	4	96	4.0
Cocklebur	16	86	15.6	*T. viride*§	4	96	4.0
Sage brush	16	86	15.6	*Pullaria* spp.	4	98	3.9
Lambs quarter	12	90	11.7	*Rhizopus* spp.	4	98	3.9
Kochia	10	92	9.8	*S. chartarum*	3	97	3.0
Sorrel	7	95	6.8				

**Values presented represent the total number of individuals (n = 102) identified by SPT to be either positive or negative to a panel of aeroallergen extracts, with the percentage frequency of positive SPTs.*
#Extracts of T. viride *and* C. globosum *prepared commercially by Greer Laboratories.*
§Extracts of T. viride *and* C. globosum *prepared commercially by Antigen laboratories.*

markers (BioRad) were used for molecular mass determinations. For Western blots, gels were electrophoretically transferred overnight at 30 V to a nitrocellulose membrane (0.2 μm; BioRad). The membrane was stained with Ponceau S (Sigma-Aldrich, St. Louis, MO) for 2 minutes. After visualizing the proteins, the membrane was rinsed with dH_2O and then destained in 0.1 M of NaOH for 10–30 seconds. After rinsing three times with dH_2O the marked membrane was blocked with 3% bovine serum albumin (BSA) in PBS and placed into a multiscreen apparatus (BioRad). The blocked membrane was washed three times with PBS/Tween 0.05% (PBST) and incubated overnight at 4°C with patient sera diluted 1:5 v/v with 3% BSA-PBST. After washing three times for 10 minutes in PBST, the membrane was incubated for 90 minutes with a 1/5000 dilution of phosphatase-labeled goat anti-human IgE (KPL, Gaithersburg, MD). The membrane was then washed three times for 10 minutes in PBST. Bound antibody was visualized by incubation with nitroblue tetrazolium and bromo-chloro-indolyl phosphate (NBT/BCIP; Roche Diagnostics, Indianapolis, IN). Color development was stopped after 35 minutes. To examine cross-reactivity of IgE to indoor fungi with other common species such as *A. alternata* and *A. fumigatus* inhibition immunoblots were used. A fungal IgE pool was created by combining equal volumes of four patients with IgE^+ sera identified in the initial Western blot analysis. The pooled sera (1:5 dilution in 3% BSA-PBST) was preincubated with 7.5 μg of either the fungal extract being tested, *A. alternata*, or *A. fumigatus* extract for 1 hour with agitation at RT. All of

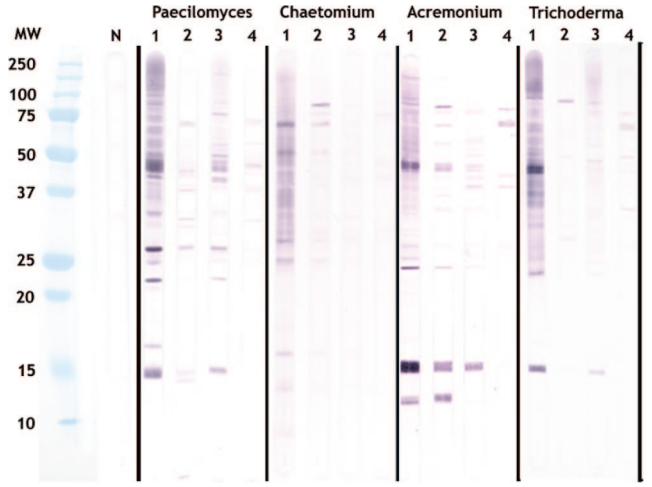

Figure 1. *Western blot showing IgE reactivity of fungal-sensitized patients to extracts from indoor fungi. The fungal extract applied to the gel is indicated at the top of each panel. The reactivity of four fungal SPT$^+$ patients (lanes 1–4) against each extract is shown for* P. variotii *(panel 2),* C. globosum *(panel 3),* A. strictum *(panel 4), and* T. viride *(panel 5). Prestained molecular weight markers are shown in panel 1. No immunostaining was observed using nonatopic control serum against the* T. viride *extract (lane N, panel 1) or the other extracts (data not shown).*

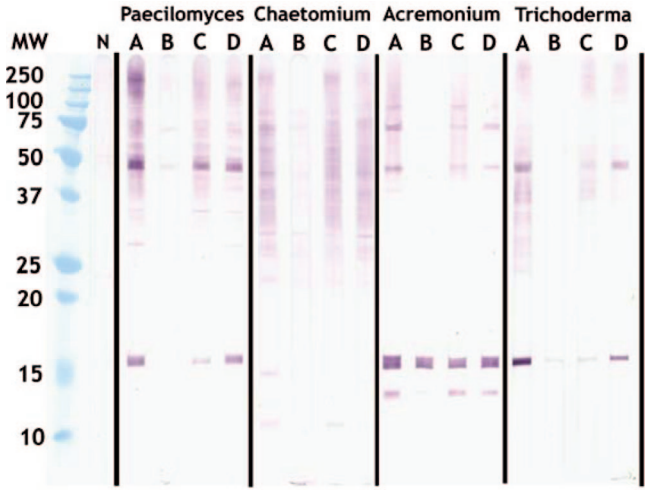

Figure 2. *Inhibition of pooled human antifungal IgE. Equal volumes of sera from the four patients shown in Fig. 1 were pooled and used for the inhibition studies. Each serum was inhibited with 7.5 μg of fungal extract for 1 hour before addition of the sera to the membranes. For each fungal panel, A represents no inhibition, B represents inhibition using the same extract as the extract transferred to the membrane, C represents inhibition with* A. alternata, *and D represents inhibition with* A. fumigatus. *Prestained molecular weight markers are shown in panel 1. No immunostaining was observed using nonatopic control serum against the* A. strictum *extract (lane N, panel 1) or the other extracts (data not shown).*

the other steps were performed as described for the Western blot analysis.

RESULTS

Of 102 patients, 73 patients were female and 29 were male patients, with a mean age of 40.1 years (range, 8–78 years), 64 (62.7%) had allergic rhinitis, and 5 (4.9%) had asthma. A total of 51 antigens were tested and 69 patients (67.6%) reacted to at least 1 antigen (Table 1). The most common antigens eliciting a positive skin test were dust mites (39%), cats (25%), vernal grass (26%), short ragweed (26%), and redtop grass (25%; Table 1). Twenty-one patients (20.5%) tested positive to at least 1 fungal extract, the most prevalent was to *A. alternata* (11.8%, Table 1). Of patients with a positive SPT to the fungal panel, 11/21 patients showed sensitivity to one or more of the additional indoor fungal contaminants, most notably, *T. viride*, *C. globosum*, *P. variotii*, and *A. strictum* (Table 1). Four of the fungal-sensitized patients (4/21, 19%) reacted to the additional indoor fungi without reactivity to our routine fungal panel. Only 3 patients (3%) tested positive to *S. chartarum*. Differences in SPT reactivity for specific fungal extracts manufactured by different companies were observed also for *T. viride* (4%, Antigen Laboratories, compared with 8%, Greer Laboratories) and *C. globosum* (4% compared with 7%, respectively).

Immunoblotting was performed using sera from fungal SPT$^+$ individuals revealed multiple proteins ranging from ~10 to 250 kDa that were IgE reactive. Figure 1 shows that the IgE reactive proteins recognized by four separate fungal-sensitized patients varied in intensity and the bands recognized. Several bands within a species were recognized by most of the individuals, while others appeared to be unique to an individual. In addition, there were bands at ~16 and 50 kDa, strongly recognized by at least one patient (lane 1), that appear to be present in extracts from *P. variotii, A. strictum*, and *T. viride* but not in *C. globosum* (Fig. 1). Nonatopic control serum was negative toward the fungal extracts (Fig. 1).

The extent of cross-reactivity between indoor fungal extracts and *A. alternata* or *A. fumigatus* was examined by immunoblot inhibition. Figure 2 shows that the IgE reactivity was nearly completely inhibited using the soluble extract of the same species of fungi that was on the blot (lanes B) except for *A. strictum* where the 16-kDa protein was only partially inhibited. Using *A. alternata* or *A. fumigatus* extracts, partial inhibition could be observed for some protein bands. In particular, the 16-kDa band observed in *P. variotii, A. strictum*, and *T. viride* was partially inhibited with the *A. alternata* extracts (Fig. 2). The degree of inhibition of IgE binding to these fungi was significantly greater than that identified in *P. variotii* and *C. globosum* inhibition immunoblots. Interest-

ingly, the IgE binding to *A. strictum* observed as a smear of activity also was found to be inhibited by the *A. fumigatus* extract (Fig. 2). However, the degree of inhibition of IgE binding to these fungi was significantly greater than that identified in *P. variotii* and *C. globosum* inhibition immunoblots.

DISCUSSION

West Virginia has one of the highest hospitalization rates for children because of asthma when compared with the rest of the United States.[8] Studies have identified allergies to mouse, cockroach, and other insect allergens as prevalent within this region[9–11]; however, sensitization to fungi has not been reported. Increased levels of outdoor fungal aeroallergens have been associated with asthma exacerbations in large populations[12,13] and with sudden respiratory arrest in young patients with asthma with IgE to *A. alternata*.[14]

IgE sensitization to fungi that contaminate indoor environments are important causes of allergic disease in both home and work environments.[1,15] Indoor fungal growth is associated with damp environments or water intrusion[16] and requires prompt and aggressive attention to prevent extensive contamination and related allergic heath problems.[17] *Paecilomyces*, *Acremonium*, *Trichoderma*, and *Chaetomium* species frequently are cultured from indoor environments[6]; however, these fungi usually are overlooked as potential aeroallergen sources. This is partly because of the vast number of fungi that occur naturally and the diversity of allergens within different species. In the present study, we have examined the prevalence of sensitization to these indoor fungal contaminants and found SPT reactivity, in most cases, to be higher than the fungal allergens that are used in our routine fungal panel. In 19% of fungal-sensitized patients, diagnosis of fungal sensitization would have been missed without testing for these indoor species. Although a positive SPT does not necessarily mean allergic reactivity, these findings indicate the importance of testing for regionally prevalent fungal species.

Currently, no allergens have been identified or characterized for *Paecilomyces*, *Acremonium*, *Chaetomium*, or *Trichoderma* species. Using immunoblotting we observed multiple IgE reactive bands in extracts of each of these fungal species that were not inhibited by other common fungal aeroallergen sources. Additional work is necessary to identify the allergens and determine the extent of their cross-reactivity with other fungal species.

It is well known that the allergen content in fungal extracts is highly variable.[18] Variability within a species has been documented for several fungi, including *A. alternata*, *A. fumigatus*, and *Cladosporium herbarum*.[19] Different strains of these species vary greatly in composition and it is difficult to grow two consecutive cultures with similar antigenic profiles. Studies have shown that commercial extracts, where a single allergen has been assayed, may vary up to 3000-fold for *A. alternata* extracts.[20] Our results show that SPT reactivity to two different extracts of *T. viride* was highly variable and ranged from 3.8% (Antigen Laboratories) to 7.6% (Greer Laboratories). These findings further highlight the need for standardized fungal allergen extracts.

Interestingly, the prevalence of sensitization to *S. chartarum* was found to be very low (3%). The role of *Stachybotrys* in causing adverse health effects has been widely speculated; however, few studies have reported allergy to this species.[21] *Stachybotrys* requires damp environments for growth and may not release large quantities of individual spores into the environment. Sheldon *et al.*[6] found *Stachybotrys* to be present in 6% of indoor environments throughout different seasons and regions within the United States. Our results suggest that *S. chartarum* is not an important indoor aeroallergen in West Virginia.

In conclusion, this study indicates that sensitization to these indoor fungal contaminants can be detected in the atopic population in West Virginia. These fungi have been shown to cause certain respiratory and pathogenic diseases.[7] The extent that these fungi exacerbate allergic rhinitis and asthma within indoor environments of this region requires additional investigation.

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