
IgE-reactive proteins from *Stachybotrys chartarum*

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Background: *Stachybotrys chartarum* has been associated with idiopathic pulmonary hemorrhage in infants. This is thought to be mycotoxin-related. There are increasing numbers of reports linking this fungus to the indoor environment of patients with other pulmonary problems, including allergies and asthma.

Objective: Given the potential significance of this fungus as a pulmonary pathogen, this work evaluates the antigenic proteins of *S. chartarum* as to their molecular size and the prevalence of immunoglobulin (Ig)E and IgG directed against them in the general population.

Methods: *S. chartarum* was isolated from a local home. *S. chartarum* for extract production was grown on minimum salts and glucose. Plasma from 132 healthy individuals was evaluated for IgE and IgG directed against *S. chartarum* using direct and inhibition enzyme immunoassay. The number and molecular size of those proteins that were bound by IgE from pooled sera known to contain IgE to *S. chartarum* were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblotting.

Results: Enzyme immunoassay indicated 65 of 132 (49.2%) sera tested contained IgG against *S. chartarum* and 13 of 139 (9.4%) sera tested contained IgE against *S. chartarum*. Pooled sera identified two IgE-binding proteins from extracts of *S. chartarum* spores and mycelia. These proteins are 34 and 52 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblot.

Conclusions: We conclude sensitivity to *S. chartarum* is potentially much more widespread than previously appreciated. This fungus may impact the asthmatic and allergic population through both immunologic and toxic mechanisms. Its significance in the milieu of allergenic fungi may need to be re-evaluated.

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INTRODUCTION

Stachybotrys chartarum is a fungus with wide geographic distribution. It grows well on materials with high cellulose content such as sheetrock or jute-backed carpet.^{1,2} It is often found in homes with a history of flooding or water damage.³ *S. chartarum* conidia are enveloped in a droplet of moisture when grown in the laboratory. Spores become powdery as the substrate dries.⁴ They become aerosolized if disturbed by wind or vibration.⁵

Exposure to *S. chartarum* has been associated with human illness in a number of studies.^{5–7} Often the mechanism is not immunologic.^{8,9} Evidence that patients seen by allergists for respiratory related symptoms are disproportionately exposed to these spores is increasing.^{3,10} For this study, we hypothesize that *S. chartarum* exposure is more common than is presently appreciated and that exposed, atopic individuals will produce immunoglobulin (Ig)E to at least one *S. chartarum* protein. To test this hypothesis, we determined the frequency of antibodies directed against *S. chartarum* in random serum samples taken from the general population. Immunoblotting and inhibition immunoblotting are used to

demonstrate the presence and approximate molecular size of IgE-binding proteins contained in *S. chartarum* extracts.

MATERIALS AND METHODS

Selection of Sera

General population plasma specimens for this study (n = 139) were obtained from tagged ends of blood donor units that were to be discarded. Sera for immunoblots were pooled (1 mL each) from 15 known atopics (skin prick test positive to at least one mold, usually *Alternaria*) who had confirmed or strongly suspected exposure to *S. chartarum* based on the results of a home inspection in which *S. chartarum* was found. Other fungi found in these homes included *Penicillium/Aspergillus*, *Cladosporium*, ascospores, *Alternaria*, *Periconia*, *Stachybotrys*, basidiospore, chaetomium, smut, torula, sordaria, and rust.³ Aliquots of pooled sera were stored frozen until use. Plasma was used for the IgE and IgG screening and serum was used for the immunoblots. All clinical studies were performed after obtaining approval from the pediatrics institutional review board of the University of Missouri-Kansas City.

Growth and Extraction Methods

S. chartarum was isolated from a home environmental sample collected in Kansas City, MO. The isolate was grown at 24° C for 4 weeks without shaking on a chemically defined sucrose medium.¹¹ The cultures were exposed to 12-hour

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periods of alternating light and dark. Fungal mats containing both spores and mycelia were washed with acetone to remove potential toxic materials⁴ and air-dried for 24 hours at 37° C. Dried material was powdered and extracted at 4° C for 24 hours in ammonium bicarbonate (0.01 M, pH 7.5) at 10 g dry weight per 100 mL. Eluted materials were filtered for sterility, dialyzed overnight against 50 millimolar ammonium bicarbonate in a 3-kD cutoff membrane (Spectrapore, Houston, TX) at 4° C and lyophilized. The resultant preparations were stored desiccated at -20° C until used.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% vertical slab gels (BioRad, Hercules, CA) using the method of Laemmli.¹² Prestained molecular weight markers (BioRad) were included with each run. Lyophilized samples were reconstituted at 2.5 mg/mL; in Tris(hydroxymethyl) aminomethane-sodium dodecyl sulfate (pH 6.8), and 10 μ L was applied to each lane.

IgE Immunoblot

Proteins from SDS-PAGE gels were transferred electrophoretically to nitrocellulose membranes (BioRad TransBlot, BioRad) in 25 mmol/L TRIS and 192 mmol/L glycine buffer, pH 8.8, containing 20% methanol at 30 V for 2 hours.¹³ After blocking with 5% nonfat dry milk (Carnation, Los Angeles, CA) in tris-buffered saline (TBS), the blots were probed overnight with 1 mL pooled human serum in 20 mL TBS 0.5% Tween and washed in three changes of TBS-Tween. The blots next were incubated for 2 hours with 10 mL 1:1,000 alkaline phosphatase-conjugated goat anti-human IgE (Sigma, St Louis, MO), washed and developed with alkaline phosphatase substrate (Kirkegaard and Perry, Gathersburg, MD) until bands were well visualized. Immunoblots contained both ragweed and *Alternaria alternata* extracts as positive controls. To further define the specificity of human IgE containing sera for *S. chartarum*, inhibition immunoblotting was performed. Sera were preincubating with a 1:10 dilution of the 10 mg/mL *S. chartarum* extract and then used in the standard blotting procedure.

Human IgE and IgG Enzyme Immunoassay (EIA)

Polystyrene microtiter plates (Immulon-2, Dynatech Laboratories, Chantilly, VA) were coated by dissolving lyophilized extract at 10 μ g/mL in a 0.15-M bicarbonate buffer (pH 9.6) and adding 100 μ L per well overnight at 4° C. For the human IgE- and IgG-sandwich EIA, coated microtiter plates were blocked with 0.5% gelatin (Sigma) at 37° C for 1 hour. Plasma samples were diluted with phosphate-buffered saline/Tween-20/azide buffer (PTA) containing 0.5% gelatin at a rate of 1:10 for IgE determinations and 1:100 for IgG determinations. To each well 100 μ L-diluted plasma was added. Plates were incubated for 2 hours at 37° C and then washed with PTA. After washing, 100 μ L of a 1:1,000 dilution of alkaline phosphatase conjugated antibody made in goat and directed specifically against the epsilon chain of human IgE (gamma chain of human IgG in the IgG determinations;

Sigma) was added to each well. The alkaline phosphatase conjugated antibody was detected with alkaline phosphatase-substrate (Sigma). The optical density at 405 nanometers (OD405) was then determined. Controls were integrated into each individual EIA plate and included a negative (sera from a non-IgE producer), an atopic negative (sera from an atopic individual not sensitive to *S. chartarum*), and a positive (pooled sera from atopic individuals exposed to *S. chartarum*). An inhibition method was used to ensure bound IgE identified was directed to *S. chartarum* proteins only. Each plasma or serum was added to two individual wells on the plate. *S. chartarum* extract at a dilution of 100 μ g/mL was added to one of the pair of coated wells as a binding inhibitor. Sera were determined to be positive for IgE if OD values were at least three times the negative control in the uninhibited well and the OD decreased by at least 30% in the inhibited well. Sera were determined to be positive for IgG if OD values were at least 10 times the negative control in the uninhibited well and the OD decreased by at least 30% in the inhibited well.

IgE Inhibition Analysis

For inhibition analysis, microtiter plates were coated and blocked as above. *S. chartarum* extract starting at 1 mg(lyophilized dry weight)/mL was added to the top of each column and titered down the column in $\frac{1}{2}$ -log dilutions. Pooled human sera containing IgE directed against *S. chartarum* diluted 1:100 in PTA were added to each well of the column. Controls included plasma from an atopic individual not sensitive to *S. chartarum* and plasma from a nonatopic individual. Results were calculated as percentage of inhibition as compared with a well containing no inhibitor.

RESULTS

IgG and IgE EIA Results

By the strict criteria described above, 49.2% of 132 individual sera randomly obtained from healthy individuals contained IgG directed against *S. chartarum* extract. IgG sera were diluted 100-fold before testing. IgG microtiter plates were allowed to develop until positive controls exceeded 1.5 OD. Typical IgG positive sera measured >1.0 OD in the uninhibited well and <0.6 in the inhibited well. Similarly, by the criteria described above, 9.4% of 139 individual sera randomly obtained from healthy individuals contained IgE directed against *S. chartarum* extract. IgE sera were diluted 10-fold before testing. IgE-coated microtiter plates were allowed to develop until positive controls reached 1.0 OD. Typical IgE positive sera measured >0.5 OD in the uninhibited well and <0.25 in the inhibited well.

EIA Inhibition Analysis

IgE inhibition analysis using *S. chartarum* extract and pooled sera containing IgE directed against *S. chartarum* applied to the microtiter plate at 1:10 dilution (Fig 1) indicated 50% inhibition at 0.7 mg(lyophilized dry weight)/mL *S. chartarum*. We were able to achieve 54% inhibition at 1 mg/mL

Stachybotrys IgE Inhibition Analysis

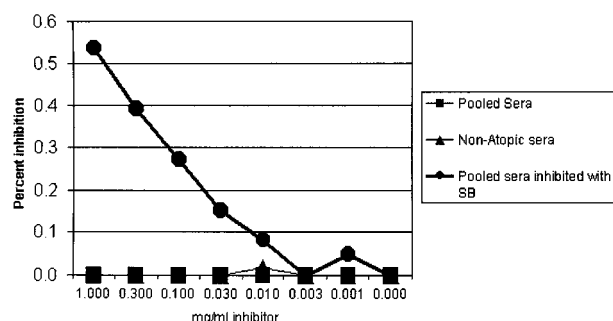


Figure 1. IgE inhibition analysis. An EIA plate coated with *S. chartarum* extract is incubated with pooled human serum known to contain IgE antibodies to *S. chartarum*. Experiments were conducted with varying concentrations of the same *S. chartarum* extract in the liquid phase to inhibit IgE-binding to the solid phase. Results are displayed as percentage of inhibition for decreasing concentrations of inhibitor. A nonatopic serum was used to control for possible proteolytic activity in the extract or nonspecific secondary antibody binding. These results support the hypothesis that the pooled sera contains IgE antibodies specific for *S. chartarum* proteins.

inhibitor. Sera from two atopic individuals not sensitive to *S. chartarum* showed no inhibition even at the highest inhibitor concentration. Sera from a nonatopic individual showed no IgE-binding.

Immunoblotting Results

IgE immunoblots using pooled sera known to have a high anti *S. chartarum* IgE content indicate that there are two primary IgE-binding areas on SDS-PAGE blots of *S. chartarum* extract (Fig 2). The weaker of these binding areas is at 52 kD and the stronger is 34 kD. The blots were probed with pooled sera containing IgE directed at *S. chartarum*, as well as IgE-containing serum from an atopic individual who is not sensitive to *S. chartarum* and serum from an individual who does not produce IgE. The blot also indicates two diffuse bands for ragweed (50 and 34 kD) and a sharp band at 30 kD for *A. alternata* are recognized by the pooled sera and the atopic individual. These bands serve as positive controls both for the blotting procedure and additional molecular weight markers.

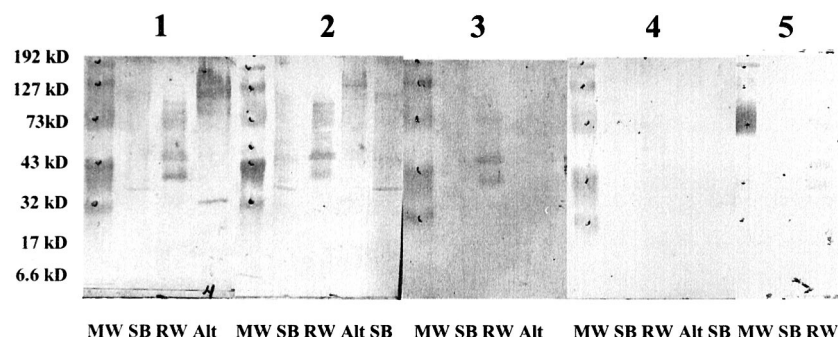


Figure 2. SDS-PAGE immunoblots of molecular weight markers (MW), *S. chartarum* extract (SB), ragweed extract (RW), and *Alternaria* extract (Alt). Blots 1 and 2 are probed with pooled sera containing IgE directed against *S. chartarum*. Blot 3 is probed with control sera from an atopic person not sensitive to fungi. Blot 4 is probed with sera from a nonatopic control. Blot 5 is probed with pooled sera inhibited with *S. chartarum* extract. The major *S. chartarum* proteins recognized are at 52 and 34 kD. The serum pools and the atopic control recognize ragweed proteins at 50 and 38 kD. The pooled sera recognize the Alt a 1 band at 30 kD, whereas atopic control does not recognize *Alternaria*.

Inhibition Immunoblot Results

IgE-binding to SDS-PAGE blots of *S. chartarum* extract was completely inhibited by the same extract added to the serum pool before exposure to the blotted proteins (Fig 2). The positive control blotting is still evident in the ragweed control bands seen on the blot. Two milliliters of IgE serum were preincubated with 2 mg *S. chartarum* extract. Inhibition of IgE against *S. chartarum* seems to be complete.

DISCUSSION

We have demonstrated that, in the general population, antibodies directed against *S. chartarum* proteins are possibly more common than previously anticipated. These proteins were identified on immunoblots. Their specificity was confirmed by inhibiting with *S. chartarum* extract both on immunoblots and EIA. This is the only report that could be found in several electronic literature searches that describes specific *S. chartarum* proteins against which atopic individuals produce IgE. Most publications concerning this fungus deal with its toxic aspects.^{2,4,10,12,14} Because of the toxic reputation of this fungus, allergists may have avoided routine skin testing. However, other fungi such as *Alternaria*, *Cladosporium*, and *Aspergillus* have known toxic potential.^{15,16} Asp f 1 is known to be an allergen and a toxin.¹⁷ It is not unexpected that humans would develop an IgE response to toxic fungal proteins.

S. chartarum was initially associated with cases of pulmonary hemorrhage in a series of infants in Cincinnati.^{18,19} But this has since been revisited and currently the association is less certain.²⁰ In our investigations we find evidence of *S. chartarum* either in the air or in tape samples taken from active growth or dust in more than 30% of homes tested on referral from a pediatric allergy/immunology clinic.³ Very often the discovery of spores occurs some time after a water leak when spores become powdery and airborne dispersal occurs.⁵

The high percentage of healthy individuals who produce either IgG or IgE antibodies directed against *S. chartarum* proteins was unexpected. The criteria for a positive assignment were set with respect to known negative and positive sera. The criteria for IgG were set to avoid false positives from nonspecific IgG-binding. The 30% OD reduction upon

inhibition was set to provide specificity for the identification, keeping in mind that we were only able to produce 54% inhibition on EIA plates even when very high inhibitor concentrations were used (Fig 1). The anti-*S. chartarum* IgE in 9.4% of the general population in this study compares with 7% for house dust, 7% for *Alternaria*, and 12% for ragweed.²¹ At this time we can not determine whether these responses were caused by *S. chartarum* exposure or by exposure to cross-reacting proteins from other fungal genera.

Symptoms reported to be associated with acute *S. chartarum* exposure include dermatitis, pain and inflammation of the mucous membranes, bloody rhinitis, cough, and fever.²² Patients in an allergy clinic often express these symptoms. The reader should be cautioned that just because a person produces IgG or IgE to *S. chartarum*, it does not mean they have been exposed to toxin. Similarly, IgG and even IgE directed against *S. chartarum* may be the result of exposure to cross-reacting proteins from other fungi.

There have been previous reports of fungal-directed IgE in the sera of persons who work in sick buildings^{23,24}; however, *S. chartarum* was not among the fungi tested. *S. chartarum* serology is commercially available from at least one laboratory in the United States (IBT Reference Laboratory, Lenexa, KS).²⁵

The molecular sizes of the two IgE-binding proteins identified in this work are not unusual. Most protein allergens are of low to moderate molecular weight. The not-reduced *Alternaria* Alt 1 protein used as a control on Figure 2 has been reported to be from 28 to 31 kD by different authors.^{26,27} Of the two proteins seen in the ragweed control on Figure 2, the lower is consistent with the 38-kD isoform of the Amb 1 allergen.²⁸ The fainter of the two IgE-binding proteins identified from *S. chartarum* extract is 52 kD. This is the same approximate range as the enolase protein allergens identified in several fungal extracts.²⁹ There is insufficient evidence at this time to identify the molecular purpose of either of these two proteins.

CONCLUSION

The toxigenic potential of *S. chartarum* may have an impact on the present dearth of information regarding the allergenic potential of this fungus. Even though this fungus is currently much in the news, we know only one current supplier of *S. chartarum* skin testing extract (Antigen Labs, Liberty, MO). Although there is evidence that not all strains produce toxin, and that toxin synthesis often does not occur,⁷ physicians may be reluctant to test with potentially toxic substances. Clearly, much work remains to be completed before either the toxic or allergic potential of this fungus is well described.

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