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Antibodies against *Stachybotrys chartarum* extract and its antigenic components, Stachyhemolysin and Stachyrase-A: A new clinical biomarker

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Summary

Background:

IgG and IgE antibodies against *Stachybotrys* extract have been reported in allergic patients and residents of water-damaged buildings. Detection of these antibodies in blood was partially attributed to cross-reacting proteins from other fungi. There is a need for a specific method to detect antibodies against characteristic components of *S. chartarum*.

Material/Methods:

We measured IgG and IgE antibodies against *Stachybotrys* hemolysin and proteinase-Stachyrase-A by ELISA and ELISA-inhibition techniques.

Results:

Of 50 reference sera with IgE greater than 500 IU/ml and positive against different mold extracts used in this study, significant elevation in IgG or IgE antibodies against *S. chartarum* extract was present in 25 and 21 specimens. Of these specimens 20 (80%) and 10 (40%) were positive for IgG anti-*Stachybotrys* hemolysin and anti-Stachyrase-A, while 8 out of 21 sera (38%) and 17 out of 21 (81%) specimens were positive for IgE anti-*Stachybotrys* hemolysin and anti-Stachyrase-A respectively. Inhibition studies using *Stachybotrys* hemolysin and Stachyrase-A at a concentration of 50 µg/ml prevented binding of anti-*Stachybotrys* to *S. chartarum* extract.

Conclusions:

Detection of IgG as well as IgE antibodies against *Stachybotrys* hemolysin and Stachyrase-A and inhibition of anti-*Stachybotrys* binding to *Stachybotrys* antigens indicate that *Stachybotrys* hemolysin and Stachyrase-A are two major antigenic components of *S. chartarum* extract, which can be used in antibody assays. Measurement of antibodies against these characteristic components of *S. chartarum* may be considered for demonstration of exposure and possibly allergy to the fungus.

key words:

Stachybotrys • Stachyhemolysin • Stachyrase-A • antibodies • ELISA

Abbreviations:

ELISA – Enzyme-Linked Immunosorbent Assay; **EIA** – Enzyme Immunoassay; **S.** – *Stachybotrys*; **kDa** – Kilodalton; **SDS** – Sodium Dodecyl Sulfate; **PBS** – Phosphate Buffer Saline; **CNBr** – Cyanogen Bromide; **I.U.** – International Units; **IgG** – Immunoglobulin-G; **IgE** – Immunoglobulin-E; **BSA** – Bovine Serum Albumin; **HSA** – Human Serum Albumin

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BACKGROUND

Individuals allergic to one mold frequently have skin test reactions elicited by extracts of other genera of fungi [1]. This probably results from the presence of shared antigenic determinants or separate immunologic responses to antigens of different fungal species [2]. *S. chartarum* is a potentially toxigenic fungus that has been reported to be associated with human health concerns such as pulmonary hemosiderosis in infants, [3–7] and nasal bleeding in adults [8–10]. Because of the toxic reputation of *Stachybotrys*, allergists may have avoided routine skin testing to determine potential allergenic reactions to this fungus. In this respect, there is only one published report describing specific *S. chartarum* proteins against which atopic individuals produced IgE [11]. Utilizing EIA these authors reported that 65 of 132 (49.2%) sera contained IgG and 13 of 139 (9.4%) had IgE against *S. chartarum* reactive proteins. Based on these observations, they concluded that sensitivity to *S. chartarum* is potentially more widespread than previously accepted. This fungus may impact the asthmatic and allergic population through both immunologic and toxic mechanisms [11].

We have confirmed these findings by examining IgE and other isotype antibodies not only against *Stachybotrys* extracts but also against mycotoxins produced by fungi in sera of 40 controls and 40 symptomatic patients exposed to *S. chartarum* in a water-damaged building [12–14]. Significant elevation of specific IgE antibodies against *Stachybotrys* extract, Satratoxin and trichothecene were detected in 7.5–10% of controls, but in 20–27.5% of symptomatic patients [13]. However, due to possible antigenic sharing between different fungi found in these studies, detection of IgG and IgE antibodies against *S. chartarum* may also arise because of cross-reacting antigens of other fungi [11–14]. Therefore, there is a need for identification of components characteristic of this fungus to be used in specific detection methods. Strains of *S. chartarum* produce several mycotoxins, immunosuppressive agents, hemolysins, and proteinases, some of which are involved in tissue destruction [8–10,15,16]. Two potential antigens have been identified from a toxic strain of *S. chartarum*: Stachyrase-A, a chymotrypsin-like serine proteinase [15] and Stachyhemolysin [10,16]. Utilizing an ELISA assay, Stachyhemolysin was detected in the serum of five human adults exposed to *S. chartarum* in a water-damaged environment, while none was found in controls. Thus, detection of Stachyhemolysin in serum might provide a biomarker [17]. Based on these findings, we hypothesized that environmental exposure to Stachyhemolysin and Stachyrase-A from *S. chartarum* (molecular masses of 12 and 32 kDa) may result in antibody production against these mold antigens. In this study we report IgG and IgE antibody levels against these molecules and compared them to levels against *S. chartarum* extract by an ELISA procedure. In addition, using affinity-purified rabbit and human serum made against *S. chartarum* in the ELISA inhibition assay, we demonstrated the degree of cross-reaction between these antigens. Identification of immunologically important antigens (e.g. Stachyhemolysin and Stachyrase-A) and the measurement of IgE and IgG antibodies against them may result in a more specific diagnosis of allergy and better management of patients exposed to toxic strains of *Stachybotrys*.

MATERIAL AND METHODS

Human reference sera

Fifty reference sera with IgE higher than 500 I.U./ml that were positive for IgE against different mold extracts obtained from Diagnostic Products Corporation (Los Angeles, CA) were used in this study. The individuals (37 to 65 yrs; 25F, 25M) were obtained from our data base of patients in the Houston and Los Angeles areas.

Antigens

The protocol for optimal fungal antigen extract preparation was based on earlier procedures [12–14,18]. In brief, *Stachybotrys chartarum* was obtained from the American Type Culture Collection (ATCC NO 34915), Rockville, Maryland. The mold was first cultured in 2% malt extract agar for 8 days at 25°C, after which spore suspensions were prepared in 0.1M PBS pH 7.4 containing 0.05% tween 20. One milliliter of each spore suspension was inoculated into 100 ml of 2% malt extract broth in glass bottles; the cultures were incubated for 10 days on a shaker at 25°C. Mycelia were separated from the broth by centrifugation at 2,000 g for 20 minutes, dried in a vacuum dryer, and stored at –70°C. Dried mycelium containing spores were suspended at 50 mg dry-weight/ml in 0.1M PBS pH 7.5 containing 0.02% phenyl methyl sulfonyl fluoride (PSF) and 0.02% sodium azide. Mycelium suspension was sonicated for 5 seconds at output of 70% using Virsonic 50, the Virtis Company, Gardiner, NY. The sonication step was repeated ten times for maximum cell disruption and thereafter the suspension was kept on a shaker for 24 hours at 4°C. After centrifugation at 4,000 g, the supernatant was dialyzed at a molecular cut-off of 2 kDa against PBS at 4°C for 24 hours, lyophilized and stored at –70°C. For quality control and reproduction of antigenic preparation of these mold extracts, 20 mg of each was dissolved in 1 ml of 0.01M PBS, the protein content was determined [19] and its components were analyzed by 15% SDS gel electrophoresis. The protein contents of our preparations were compared to the same mold-allergenic extracts obtained from Antigen Laboratories, Inc, Liberty, Missouri. Several more protein bands were present in our preparation compared to the allergenic extract of Antigen Laboratories; however a few bands were present in the allergenic extracts and not in ours. Consequently, we mixed our antigenic preparations with allergenic extract from Antigen Laboratories in a ratio of 1:1 and used them in the ELISA assay.

Stachybotrys hemolysin (Stachyhemolysin)

Stachybotrys hemolysin was purified as previously described [10,16]. Briefly, after 10 days growth in malt extract broth the culture supernatant was centrifuged in a Millipore Centricon plus 80 filter apparatus with a molecular mass cutoff of 30 kDa (Millipore, Bedford, Mass.) at 4,000 g for 15 min. The concentrate was recovered according to the manufacturer's instructions and then separated by DEAE-cellulose ion-exchange chromatography. Fractions of five-drops each were collected and 10 µl of each fraction was plated on sheep blood agar, and hemolysis was observed at 48–72 hours. The five most hemolytically active fractions were placed in dialysis tubing with a molecular cutoff of 3 kDa and dialyzed over 48 hours against 0.01M PBS pH 7.2. Protein electrophoresis using 7–15% acrylamide and 1% so-

dium dodecyl sulfate was performed. The gel was run at 60 v for five hours and stained with Coomassie Blue.

Stachyrase-A

The Stachyrase-A N-terminal amino acid sequence of Stachyrase-A described earlier [15] was synthesized by Biosynthesis Inc. (Lewisville, TX).

Cytotoxicity testing

Stachybotrys extract Stachyhemolysin and Stachyrase-A were tested for cytotoxicity by measuring the rate of apoptosis using kit Apo-BrdU (Phoenix, San Diego, CA) with continuous human B lymphoblastoid cell line ATCC CRL-8885. Cells were added at a concentration of 10^4 cells/well in C-RPMI with or without different dilutions of hemolysin. The plates were incubated for three days at 37°C in a humidified environment in the presence of 5% CO₂. After incubation, cells were harvested, washed, fixed, and then stained and analyzed by flow cytometry for the degree of DNA strand breaks.

Antibodies

Antibodies against *Stachybotrys* extract were prepared in New Zealand white rabbits according to standard procedures by Biosynthesis Inc. (Lewisville, TX). The immunization protocol conforms to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publication No. 85-23, revised 1985) and was approved by the institutional animal care and use committee. 2 ml of blood was drawn from 8 different rabbits of 3 months age and used as pre-immunization specimens.

Two different rabbits were injected every other week with 1 mg of mold extract in complete Freund's adjuvant. During a period of six months each rabbit received 12 different injections: six injections with commercial extracts and six injections with in-house preparation. Four weeks after the last injections, blood was collected, immunoglobulins were precipitated and purified by affinity chromatography on protein-A sepharose.

Affinity purification

Cyanogen bromide-activated sepharose 4B (Sigma) was washed with 0.3 M HCl, mixed with 10 mg/ml mold extract in 0.1M of bicarbonate buffer pH 9.6, per gram sepharose. The mixture was kept on the stirrer for 60 min at R.T. and washed in alternating 0.1M nonacetate/nonborate three times and blocked with 3% BSA. The material was then put into an affinity column (Biorad, Hercules, CA) and washed extensively with 0.1M PBS. For purification of antibodies and immunoglobulins, 5 ml of human and rabbit anti-*Stachybotrys* were dialyzed against PBS and then added to the affinity column filled with *Stachybotrys* antigens bound to sepharose 4B. After 1h incubation at R.T., the antibody passed through the affinity column and samples were collected by gravity. The protein content of the effluent was monitored continuously at 280 nm. Once the O.D. 280 returned to baseline, the bound antibody was eluted with 0.1M glycine, pH 3.0, into 0.1M Tris pH 11.0 in order to minimize the exposure of the antibody to acid. The effluent was dialyzed against 0.01M PBS pH 7.2, concentrated to its original volume, measured by ELISA for the lev-

el of IgG and IgE against *Stachybotrys* extract, and kept at -70°C until used in immunoassays.

ELISA

The levels of IgG and IgE antibodies against antigens of *S. chartarum* in human sera and in rabbits before and after immunization were analyzed by indirect ELISA. Microtiter plates were coated with 0.1 ml of either HSA in duplicate, which served as controls, or with *S. chartarum* extract, Stachyhemolysin and Stachyrase-A at a protein concentration of 10 µg/ml. After incubation, washing and blocking with 2% BSA, 0.1 ml of human serum at optimal dilution of 1:2 for IgE and 1:100 for IgG in serum diluent buffer (2% BSA in 0.1 M; PBS plus 0.01% tween 20) were added into the quadruplicate wells of the plates. Plates were incubated at 37°C for 2 hours for IgG and 24 hours for IgE and washed three times with PBS tween 20. Then 0.1 ml of affinity-purified goat anti-rabbit and anti-human IgG (γ) or IgE (ε) light chain-specific, conjugated with alkaline phosphatase at dilutions of 1:1,000 were added and incubated at 37°C for one hour. After repeated washing and addition of 0.1 ml of paranitrophenylphosphate substrate, color development was measured. Intensity of color was measured spectrophotometrically at 405 nm after incubation for 30 minutes and addition of stop solution. For each specimen the ELISA background reading of wells coated with HSA was automatically subtracted from wells coated with mold antigens. The background reading of wells coated with non-specific antigen (HSA) reacted with the serum and all other reagents was less than 12% of the absorbencies of the wells coated with mold antigens. Optical densities higher than 4 times the background was considered positive.

Cross-reaction and ELISA inhibition

The ELISA inhibition method was used to determine cross-reactivity between different mold antigens and to ensure the degree of human and rabbit IgG and IgE anti-*Stachybotrys* binding to *Stachybotrys* extracts in solid phase, and to measure the inhibition of this binding by *Stachybotrys*, Stachyhemolysin and Stachyrase-A.

For this inhibition study, microtiter plates were coated with *Stachybotrys* and blocked as above. 100 µl of serum diluent buffer was added to all wells. *Stachybotrys* extract, Stachyhemolysin and Stachyrase-A, each starting at 50 µl/ml, were added to the second row and titrated down the column in 1/2 log dilutions; 100 µl of rabbit anti-*Stachybotrys* was then added to all wells of the column. After incubation for 1 hr. at R.T., addition of enzymes labeled anti-rabbit or anti-human IgE or IgG, repeat of incubation, washing, and substrate, the color development was measured at 405 nm. Results were calculated as percentages of inhibition in antigen/antibody reaction as compared with wells containing no antigen (inhibitor) in the liquid phase. In addition, unrelated antigens of *Alternaria* extract were used as a control in the inhibition assay.

RESULTS

Induction of apoptosis

For measurement of cytotoxicity induced by *Stachybotrys* extract, Stachyhemolysin and Stachyrase-A, percent apopto-

sis of human B cell line was measured. The mean $\pm$ S.D. of three different experiments are shown (Figure 1). The control medium alone had an apoptosis of $4\pm2\%$. Increasing concentrations (10, 50 and 100 $\mu\text{g/ml}$) of *Stachybotrys* extract produced killing of 6 ± 1 , 14 ± 5 and $21\pm7\%$ of the B-cells. Similarly, different concentrations of Stachyhemolysin caused 8 ± 2 , 16 ± 9 and $28\pm9\%$ killing, respectively. The apoptosis produced by 50 and 100 $\mu\text{g/ml}$ of *Stachybotrys* extract and Stachyhemolysin was significantly increased over control medium ($p<0.0001$). In comparison to medium alone, induction of apoptosis by Stachyrase-A at all concentrations was not significant (Figure 1). These results showed that only *Stachybotrys* extracts and Stachyhemolysin, but not Stachyrase-A, have cytotoxic activity.

IgG and IgE ELISA

ELISA demonstrated that significant numbers of reference sera had elevations in IgG antibody against *Stachybotrys* extract, Stachyhemolysin, and, to a lesser degree, against Stachyrase-A (Figure 2). At optical densities of 4 times the background, positive IgG antibodies were found as follows: 1) 25 out of 50 specimens (50%) had anti-*Stachybotrys* antibodies; 2) 40% were positive for anti-*Stachybotrys* and anti-Stachyhemolysin; 3) 20% were positive for anti-*Stachybotrys* and anti-Stachyrase-A; and 4) 18% were positive for all three tested antigens (Figure 3). 4 specimens with elevated IgG antibody against *Stachybotrys* did not show any levels of IgG antibody against either Stachyhemolysin or Stachyrase-A. Also, an additional 4 specimens with IgG elevation against either Stachyhemolysin or Stachyrase-A were negative for *Stachybotrys* extract (see Figure 2).

Levels of IgE antibodies measured against *Stachybotrys* extract, Stachyhemolysin and Stachyrase-A were different from IgG (Figure 3). The distribution of IgE antibodies were as follows: 1) 21 out of 50 (42%) were positive for IgE anti-*Stachybotrys*; 2) 16% had simultaneous elevation in IgE antibodies against *Stachybotrys* and Stachyhemolysin; 3) 17 out of 50, or 34% were positive for *Stachybotrys* and Stachyrase-A; 4) 14% IgE antibodies against all three antigens (Figure 3). Two specimens were found to be elevated for IgE anti-Stachyhemolysin and Stachyrase-A, but negative for *Stachybotrys* antibody (Figure 2). Finally, 6 specimens positive for IgG and IgE against Stachyhemolysin and Stachyrase-A, but not reactive with *Stachybotrys* extract, shown in this study, may indicate that Stachyhemolysin and Stachyrase-A are major immunologically important antigens and allergens of *Stachybotrys chartarum*.

ELISA inhibition

Affinity-purified rabbit IgG and human IgE anti-*Stachybotrys* extract binding to *Stachybotrys* antigen in microtiter wells was inhibited by the same extract (50 $\mu\text{g/ml}$) added to the serum before exposure to the antigen-coated wells (Figure 4). *Stachybotrys*, Stachyhemolysin and Stachyrase-A (50 $\mu\text{g/ml}$) produced 74%, 48%, and 12% inhibition of IgG anti-*Stachybotrys* binding to *Stachybotrys* extract. ELISA inhibition dose-response curves were obtained with homologous antigen (*Stachybotrys* extract) and pure antigens (Stachyhemolysin, Stachyrase-A), or with control extract (*Alternaria*). Data presented in Figure 5 shows that a concentration of 12,000 ng/ml of homologous antigens caused

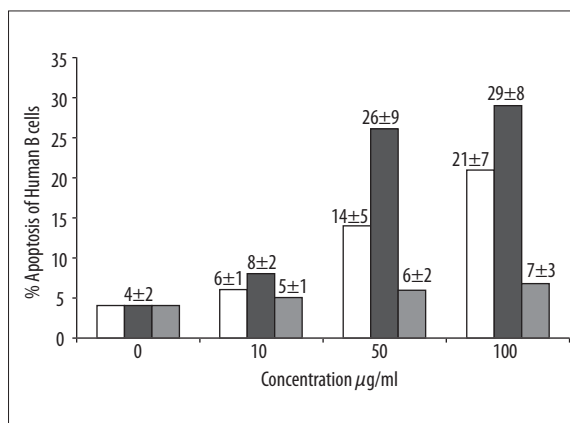


Figure 1. Induction of human lymphoblastoid cells by different concentrations of *Stachybotrys* extract $\square$, Stachyhemolysin $\blacksquare$ and Stachyrase-A $\square$. Mean $\pm$ S.D. of three different experiments.

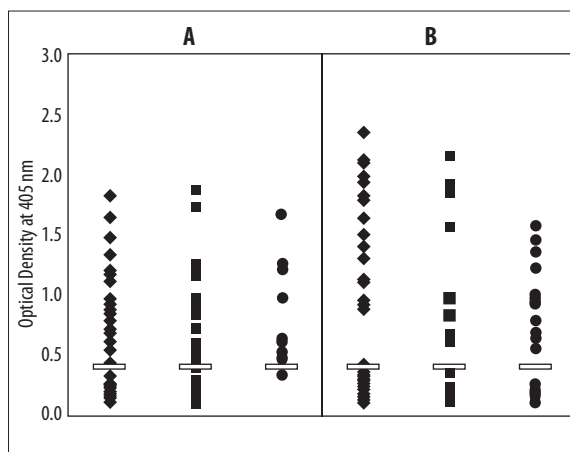


Figure 2. Scattergram of serum level of IgG (A) and IgE (B) antibodies against *Stachybotrys* extract $\blacklozenge$, Stachyhemolysin $\blacksquare$, and Stachyrase-A $\bullet$, measured by ELISA. At 4 S.D. above the control values or 0.4 O.D. antibody levels were considered significant.

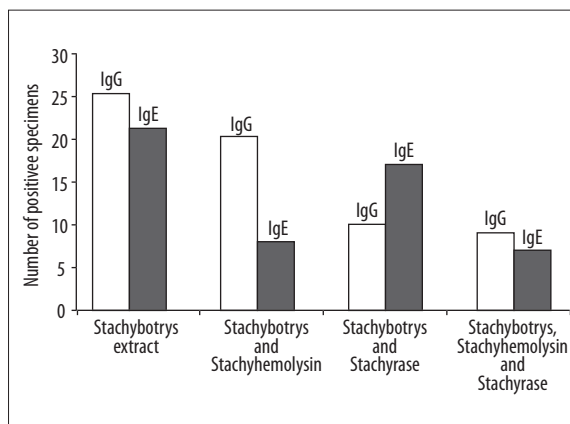


Figure 3. Number of specimens out of 50 that tested positive for IgG $\square$ and IgE $\blacksquare$ antibodies against *Stachybotrys* extract; *Stachybotrys* + Stachyhemolysin; *Stachybotrys* + Stachyrase-A; *Stachybotrys* + Stachyhemolysin + Stachyrase-A simultaneously at 4 S.D. above the controls.

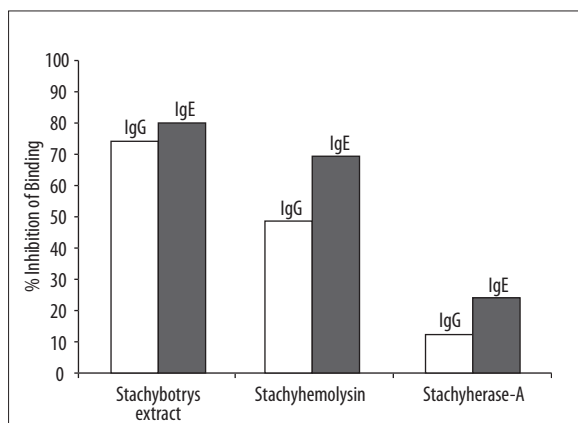


Figure 4. Inhibition of affinity-purified rabbit IgG □ and IgE ■ anti-*Stachybotrys* binding to *Stachybotrys* extract in solid phase by 50 µg/ml of: *Stachybotrys* extract, Stachyhemolysin; and Stachyrase-A.

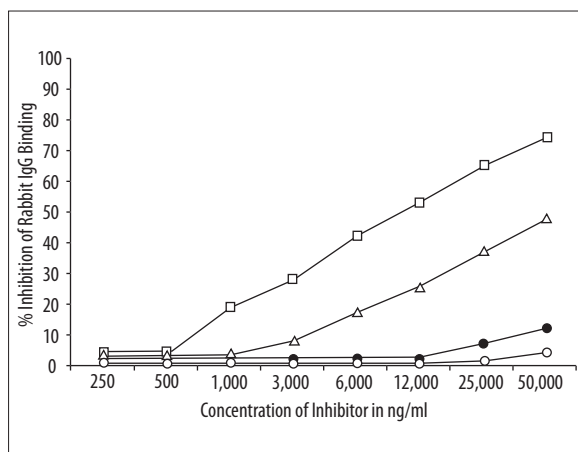


Figure 5. IgG ELISA inhibition demonstrating the extent of cross-reactivity between *Stachybotrys* extract □, Stachyhemolysin △, Stachyrase-A ●, and unrelated fungal extract (*Alternaria*) ○. Different fungal extracts of varying concentrations were used in liquid phase as inhibitors of affinity-purified antibodies.

50% inhibition of antigen-antibody binding, and the same concentration of Stachyhemolysin resulted in about 25% inhibition. With Stachyrase-A alone, a concentration of 50,000 ng/ml caused about 12% inhibition of IgG anti-*Stachybotrys* binding to *Stachybotrys* extract. *Alternaria* extract at all concentrations did not cause any significant inhibition of this antibody-antigen reaction (Figure 5). There was low ($\leq 12\%$) inhibition of rabbit IgG anti-*Stachybotrys* binding to *Stachybotrys* extract with 50,000 and 25,000 ng of Stachyrase-A, but non-significant inhibition with *Alternaria* extract (Figure 5).

The inhibition of IgE anti-*Stachybotrys* binding to *Stachybotrys* by the homologous extracts or antigens was more significant than IgG inhibition (Figure 4). Percent inhibition of IgE anti-*Stachybotrys* binding to *Stachybotrys* extract was 80, 69 and 23% when *Stachybotrys* extract, Stachyhemolysin or Stachyrase-A was present in the liquid phase (Figure 4). Dose response inhibition of human IgE binding to the same extract with all 3 antigens and control mold (*Alternaria*) is shown in Figure

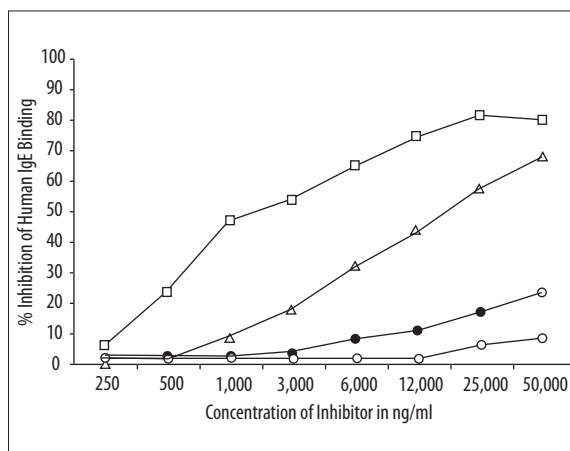


Figure 6. IgE ELISA inhibition demonstrating the extent of cross-reactivity between *Stachybotrys* extract □, Stachyhemolysin △, Stachyrase-A ●, and unrelated fungal extract (*Alternaria*) ○. Different fungal extracts of varying concentrations were used in liquid phase as inhibitors of affinity-purified antibodies.

6. Similar to the rabbit IgG inhibition, increased concentrations of *Stachybotrys* extract and Stachyhemolysin in liquid phase caused significant inhibition (up to 69–80%) in ELISA optical densities. In relation to Stachyrase-A, only concentrations of 6,000 ng/ml or greater resulted in inhibition of IgE anti-*Stachybotrys* binding to *Stachybotrys* extract from 8–23%. At the highest concentration (50,000 ng/ml) the non-related mold extract (*Alternaria*) resulted in only 8% inhibition of IgE anti-*Stachybotrys* extract (Figure 6).

DISCUSSION

Strains of *S. chartarum* produce several potentially toxic compounds, e.g. satratoxins, Stachyhemolysin, Stachyrase-A, spirocyclic drimanes, siderophores [8,15,16,20]. Stachyhemolysin causes hemolysis of red blood cells [8,16], while trichothecenes, including satratoxins, induce apoptosis [21–23]. In this study we have shown that our *Stachybotrys* extract and pure Stachyhemolysin caused a significant increase in apoptosis of B cells *in vitro* (Figure 1). The increased apoptotic activity was greatest for Stachyhemolysin (between 26 to 29%) when compared to the extract (14 to 21%). Trichothecenes, including satratoxins G and F, induce apoptosis by activation of apoptic pathways [21,23]. Thus, induction of apoptosis by the *Stachybotrys* extracts would be expected because it may contain mycotoxins, spirocyclic drimanes and other toxic compounds. Such activity by Stachyhemolysin has not been previously reported, and therefore may be an additional cytotoxic mechanism of this protein. Additional work is needed to determine the mechanism by which Stachyhemolysin induces apoptosis.

It is assumed that IgG and even IgE directed against *S. chartarum* may be the result of exposure to cross-reactive proteins from other fungi genera [11–14]. For this reason, we chose three antigen sources, *Stachybotrys*, Stachyhemolysin Stachyrase-A, for measurement of IgG and IgE in the blood of individuals with mold sensitivity. We recently reported IgG, IgM and IgA antibodies against several molds including *S. chartarum* and Satratoxin H in the blood of 500 healthy

controls, random patients and 500 individuals with known exposure to molds. The antibodies against mold antigens were significantly greater in the mold-exposed patients than in the other two groups [12–14]. In these studies we also demonstrated IgE antibodies against *S. chartarum* extract in up to 27.5% of symptomatic mold-exposed patients versus 10% in controls. Furthermore, Barnes et al [11] have shown that 49.2% of general population sera had IgG and 9.4% had elevation in IgE antibodies against *S. chartarum*. Two IgE binding proteins (M.W. 34 and 52 kDa) were identified, with the 52 kDa being similar to enolase protein allergens. In this study we set criteria for IgG and IgE at 0.4 O.D. or four times the background in order to avoid false positives from non-specific IgG- and IgE-binding. Based on this criterion, we found that 50% and 42% of specimens were significantly elevated for IgG and IgE against *S. chartarum* extract respectively (Figures 2,3). Thus, it is concluded that exposure and sensitivity to *S. chartarum* is probably much more widespread than previously appreciated. It appears that about 10% of the general population and 27.4% to 42% of symptomatic mold exposed individuals have IgE antibodies to *S. chartarum* antigens.

We have demonstrated that in reference human sera with elevated total and specific mold IgE, that significant levels of IgG and IgE antibodies against *S. chartarum* extract and two antigens (Stachyhemolysin and Stachyrase-A) are present. Specificity and cross-reactivity of these antibodies were confirmed by ELISA inhibition using affinity-purified human and rabbit serum. In comparison to *Stachybotrys* extract presence in the liquid phase and inhibition of anti-*Stachybotrys* binding to *Stachybotrys* antigens in the solid phase, Stachyhemolysin, but not Stachyrase-A, was able to inhibit this antigen-antibody reaction significantly (Figure 4). Thus, the IgG and IgE antibodies against *Stachybotrys* extract appear to contain increased levels of anti-Stachyhemolysin immunoglobulins.

Stachyhemolysin at 371 µg/ml has been demonstrated in human serum following exposure to *S. chartarum*, whereas none was found in controls [17]. It is released by conidia in the lungs and absorbed into the blood [24] and apparently accumulates over time from either respired conidia or other sources, e.g. particulate matter <0.03 microns [17,20]. Its toxicity is similar to Group B *Streptococci* hemolysins [25]. Based on all these characteristics and that antibodies against bacterial hemolysins are produced, we looked for IgG and IgE Stachyhemolysin antibodies in the *Stachybotrys* extract. IgG anti-Stachyhemolysin was present in 20 out of 25 (80%) of the sera that had the presence of IgG antibodies against *S. chartarum* extract (Figure 3). Furthermore, 21 out of 50 specimens (42%) were positive for IgE antibody to *S. chartarum* extract, 8 out of 21 (38%) had elevated IgE antibody against Stachyhemolysin as well (Figure 3). Thus, antigenicity of Stachyhemolysin is leading to the production of at least IgG and IgE antibodies to the protein.

Another antigen of *S. chartarum* is the proteinase Stachyrase-A, which is involved in tissue destruction and has been sequenced [15]. Therefore, the N-terminal sequence of this enzyme, which represents completely pure synthetic peptide, was used for antibody measurement. The IgG antibody against Stachyrase-A peptide was positive in only 10 out of 25 (40%) of IgG *S. chartarum*-positive samples, but 17 out of 21

(81%) of IgE-positive specimens (Figure 3). This difference in rate of IgG versus IgE produced against Stachyhemolysin and Stachyrase-A is an indication of separate immunologic responses (IgG versus IgE) to specific antigens of different or the same fungal species [1]. In a different study, it was shown that *Epicoecum* and *Alternaria* antigens elicit different patterns of recognition by human IgE and IgG. For this reason, in studies of cross-reactivity between inhalant allergens, the use of a class of antibody that is presumed to be of clinical significance is recommended [18]. The high percentage of sera positive for IgG anti-Stachyhemolysin but with high IgE against Stachyrase-A in our study is one example of involvement of different antibody classes against two different pure antigens.

However, in inhibition studies we showed that Stachyhemolysin concentration of 50 µg/ml (50,000ng/ml) in liquid phase was capable of inhibiting rabbit IgG anti-*S. chartarum* binding to *S. chartarum* extract in solid phase by 48%, and human IgE anti-*S. chartarum* binding to its antigen by 69%. In comparison, Stachyrase-A was able to inhibit IgG and IgE binding to their specific antigens by 12 and 23% respectively (Figures 4–6). A similar concentration of a different mold extract (*Alternaria*) used in the assay did not inhibit this antigen-antibody reaction to any significant event (<10%). Overall, it is quite interesting to note that 4 specimens positive for IgG anti-*Stachybotrys* did not show any levels of IgG antibody against Stachyhemolysin and Stachyrase-A. These antibodies may be the result of exposure to cross-reactive proteins of other fungi [11], which should be explored in additional studies. On the other hand, an additional 6 specimens with elevation of IgG and IgE against either Stachyhemolysin or Stachyrase-A were negative for IgG or IgE anti-*Stachybotrys* extract. These results are further indication that different immunological responses against crude or purified mold antigens may occur in different individuals. Based on two observations, we believe that Stachyhemolysin and Stachyrase-A are two major antigenic compounds of *S. chartarum*: First, IgG and IgE antibodies against purified antigens (Stachyhemolysin and Stachyrase-A) were detected in these subjects. Second, the ELISA inhibition studies indicate that the two antigens are highly specific. The overall correlation between IgG and IgE antibodies against *S. chartarum* extract, Stachyhemolysin and Stachyrase-A was 80% (40 out of 50 sera). Detection of IgG and IgE in a significant number of sera against Stachyhemolysin and Stachyrase-A may result in elimination of cross-reactive reactions and improved diagnosis and management of patients exposed to *Stachybotrys*. Further, comparison between skin testing with *S. chartarum* extract, Stachyhemolysin and Stachyrase-A is also needed in order to find the clinical efficacy of these pure and synthetic components of *S. chartarum*.

In conclusion, while the detection of IgE and IgG antibodies against Stachyhemolysin and Stachyrase-A strongly supports our hypothesis, much work remains to be completed before the full allergic potential of these pure fungal antigens is determined. Other fungi, such as *Alternaria*, *Cladosporium*, *Aspergillus* and *Penicillium* produce cytotoxins, hemolysins, proteases, enzymes and mycotoxins [15,26–30] that can be used as models of antigen-antibody specificity. Thus, it would appear that investigations on genera or species antigens and their role in the production of IgE and IgG mediated immune responses are in order. Finally, it is imperative to use

affinity purified procedures to determine the specificity of antigen-antibody reactions because of reported cross reactivity among various species of fungi [31–34].

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