Cross-Reactivity of *Aspergillus, Penicillium,* and *Stachybotrys* Antigens Using Affinity-Purified Antibodies and Immunoassay

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ABSTRACT. In this study, the author examined the cross-reactivities of *Stachybotrys chartarum, Aspergillus niger/fumigatus,* and *Penicillium notatum* with affinity-purified rabbit sera. The molds were grown for expression of maximum numbers of antigens, after which they were extracted and mixed with commercially available extracts. The mixture was used for antibody preparation in rabbits, measurement of antibody levels, and for the demonstration of the degree of cross-reactivity. Control rabbits were injected with saline, yet they produced *significant* levels of immunoglobulin G antibodies against all mold extracts tested. The author interpreted this result to mean that sera obtained from rabbits immunized with pure mold extracts likely reflected cross-reactivity with other molds. Therefore, only affinity-purified antibodies and the most sensitive immunoassay technique (i.e., enzyme-linked immunosorbent assay [ELISA]) were used for the cross-inhibition studies. The antigenic cross-reactivities were as follows: (a) between *Aspergillus* and *Penicillium,* 19.6–21.0%; (b) between *Stachybotrys* and *Aspergillus,* 8.2–8.7%; and (c) between Stachybotrys and *Penicillium,* 7.0–9.6%. The findings of this study demonstrate that cross-reactivity studies between different molds require the use of affinity-purified antibodies and a sensitive and quantitative assay with untreated antigens. With the use of such an assay, it was determined that the cross-reactivity between *Stachybotrys, Aspergillus,* and *Penicillium* was at approximately 10%, which is less widespread than previously believed.
<Key words: antibodies, *Aspergillus*, cross-reactivity, ELISA, mold, *Penicillium, Stachybotrys*>

INDOOR AND OUTDOOR airborne molds are causally related to asthma, respiratory complaints, and sensitization.[1–6] *Stachybotrys chartarum* has been linked to infant pulmonary hemosidersosis,[7,8] and multiple genera have been identified in chronic fungal rhinosinusitis.[9,10] The detection of antibodies to mold antigens is used as an adjunct in the diagnosis of allergies,[2,5,11] aspergillosis,[12–16] infectious states,[17,18] and farmer's lung disease.[19,20] Serum immunologic patterns obtained from patients may also assist in discriminating between different clinical phases of allergic bronchopulmonary aspergillosis (ABPA)[12,15,16] and paracoccidioidomycosis.[18] Until recently, little attention has been focused on the effect of cross-reacting antibodies detected in patients' sera on results obtained in enzyme-linked immunosorbent assay (ELISA) and immunoblot testing. Analyses of patients' sera in different assays have shown that *Aspergillus fumigatus* contains determinants in common with *Epidermophyton, Trichophyton, Alternaria, Cladosporium,* and *Candida.*[21–23] From a taxonomical point of view, the genus *Aspergillus* is closely related to the genus *Penicillium.*[1,24] Indeed, *Aspergillus* and *Penicillium* contain and produce galactomannans with similar immunogenic and galactofuranosyl side-chains.[25]

Rabbit anti-*Aspergillus* and anti-*Penicillium* and sera from patients with precipitating antibodies against these fungi have been analyzed by immunoglobulin G (IgG) ELISA inhibition and immunoblot.[1] Sera from patients

with precipitins against *Penicillium* gave anti-*A. fumigatus* ELISA titers in the same range as sera from patients with aspergillosis. Rabbit anti-*Penicillium* IgG also reacted with several *A. fumigatus* antigens that had molecular weights between 28 and 128 kilodaltons (kD [a measure of the molecular mass of protein]). Furthermore, the binding of IgE antibodies against *Penicillium* in the sera of patients with ABPA to *Penicillium* antigens could be inhibited by *A. fumigatus* almost as effectively as by *Penicillium*.[1] However, Brouwer[1] reported that the binding of IgE anti-*Penicillium* to *A. fumigatus* was only slightly affected by *Penicillium* antigens. Cross-reactivity among different species of the same genus, including *Fusarium* and *Alternaria,* has also been reported.[26,27]

In other studies, nonsignificant cross-reactivity among 6 basidiomycetes species—as well as *Epicoccum nigrum* with dark-spored fungi—was observed with radioallergosorbent test (RAST) immunoprint inhibition isoelectric focusing.[13,28,29] Bradford[30] used the same RAST-inhibition followed by the immunoblot technique and assessed the degree of shared allergenic determinants of *Alternaria tenuis, Aspergillus fumigatus,* and *Cladosporium herbarum*. In the *Aspergillus* RAST, there was little or no inhibition with *Cladosporium* and *Alternaria*, but there was considerable cross-reactivity between *Alternaria* and *Cladosporium* in a dose-dependent fashion.

Recently, Raunio et al.[31] attempted to characterize immunogenic components of *S. chartarum*. The components of 65 kD, 50 kD, 37 kD, and 27 kD of *S. chartarum* in immunoblotting analyses proved to be the most characteristic of this fungus.[31] The role of glycoproteins in the cross-reactivity of *S. chartarum* antigens with 10 other fungal species that grow under conditions similar to those of *S. chartarum* was also described.[31] The use of rabbit sera immunized with *S. chartarum* revealed a slight cross-reactivity with all 10 species at low concentrations (e.g., 2–20 μg/ml). At a concentration of 20 μg/ml, *Ulocladium botrytis* inhibited a 36% visualization of the *S. chartarum* component, whereas the respective values for *Cladosporium* and *Chaetomium* were 12% and 8%. Inhibition was 0% with extracts from *Aspergillus, Penicillium, Alternaria, Phoma,* and *Aureobasidium*. *S. chartarum* inhibited the binding of rabbit anti-*S. chartarum* to 50% of its own components at a concentration of 2 μg/ml; at 20 μg/ml, inhibition was complete.[31]

On the basis of many IgE and IgG ELISA tests performed in the author's laboratory (Immunosciences Lab, Inc.) on the sera of patients with mold allergy, as well as on the simultaneous detection of antibodies against *S. chartarum, P. (notatum) chrysogeum,* and *A. fumigatus* in approximately 30% of the cases, the author decided to examine whether these antibodies are cross-reactive. Moreover, the author has also observed a 100% simultaneous presence of antibodies to *A. fumigatus* and *A. niger*. For this investigation, therefore, the author chose *A. fumigatus, P. chrysogeum,* and *S. chartarum* to study the cross-reactivity of fungal antigens and the role of antifungal rabbit sera in this cross-reaction. Researchers who determine the degree of cross-reactivity between different molds can enable clinicians to separate levels of specific and nonspecific antibodies in individuals exposed to molds.

Materials and Method

Preparation of extracts. The protocol for optimal fungal antigen extract preparation was based on earlier procedures, with some modifications.[31–33] Molds (mycotoxin-producing *S. chartarum* ATCC #34915, *P. notatum* ATCC #9179, *A. niger* ATCC #1015, and *A. fumigatus* ATCC #16903) were obtained from the American Type Culture Collection (Rockville, MD). The molds were cultured in accordance with the method described previously.[33,34] Antibodies against each mold were prepared in accordance with the standard procedures proffered by Biosynthesis, Inc. (Lewisville, TX).

Rabbit immunization and antibody preparation. The immunization protocol conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication no. 85-23)[35] and was approved by the Institutional Animal Care and Use Committee of same. From each of eight 3-mo-old rabbits, 2 ml of blood were drawn and were used as preimmunization specimens. For each mold, 2 rabbits were injected every other week with 1 mg of mold extract in complete Freund adjuvant. During a 6-mo period, each rabbit received 12 different injections (i.e., 6 injections contained commercial extracts and 6 injections contained an in-house preparation of the antigens). For quality control and reproduction of antigenic preparation of these mold extracts, 20 mg of each was dissolved in 1 ml of 0.01 *M* phosphate-buffered saline (PBS), the protein content was determined, and the sample's components were analyzed by 15% sodium dodecyl sulfate (SDS) gel electrophoresis. Two rabbits served as nonimmunized controls, and they were injected with 12 different injections of saline. Blood was collected from each rabbit at 2, 4, and 6 mo after the first injection and kept at –20 °C.

Four wk following the final injections, blood was collected from each rabbit, and immunoglobulins were precipitated and purified by affinity chromatography on protein-A sepharose. NBr-activated sepharose 4B (SIGMA, St. Louis, MO) was washed with 0.3 *M* hydrochloric acid, mixed with 10 mg/ml mold extracts in 0.1 *M* of bicarbonate buffer (pH 9.6)/g sepharose. The mixture was retained on the stirrer for 60 min at room temperature and alternately washed in 0.1 *M* nonacetate/nonborate 3 times and blocked with

3% bovine serum albumin (BSA). The material was then put into an affinity column (Biorad Laboratories, Hercules, CA) and washed extensively with 0.1 *M* PBS. For purification of antibodies, 5 ml of immunoglobulins were dialyzed against PBS and then added to the affinity columns filled either with *Aspergillus, Penicillium*, or *Stachybotrys* antigens bound to sepharose 4B. After 1 h incubation at room temperature, the antibodies passed through each affinity column, and samples were collected by gravity. The protein content of each effluent was monitored continuously at 280 nm. At the time the optical density (OD) read at 280 nm, the wavelength had returned to baseline; the respective bound antibodies were then eluted with 0.1 *M* glycine (pH 3.0) into 0.1 *M* Tris (pH 11.0), thus minimizing exposure of the antibody to acid. The effluent of each column was dialyzed against 0.01 *M* PBS (pH 7.2), concentrated to the original volume, and kept at –70 °C until time of use in immunoassays.

Immunoassays. The author performed immunoprecipitation with immunodiffusion, using Petri dishes that contained 15 ml of 1.2% agarose in a barbital buffer (pH 8.6).[36] Immunoblot analysis of mold extracts with rabbit antibodies was performed as described previously.[23,37] The IgG antibody levels against mold antigens of *Aspergillus, Penicillium*, and *Stachybotrys* in rabbit sera before and after immunization were analyzed by indirect ELISA. Specifically, microtiter plates were coated with 0.1 ml of either human serum albumin (HSA) in duplicate, which served as controls, or with mold extract at a protein concentration of 10 μg/ml. Following incubation, washing, and blocking with 2% BSA, 0.1 ml of rabbit serum (dilution: 1:100) or serially diluted in serum diluent buffer (2% BSA in 0.1 ml; PBS plus 0.01% Tween 20) was added into the quadruplicate wells of the plates. Following incubation, washing, and addition of a 2nd antibody and substrate, color development was measured. The last dilution of serum that provided an OD of twice the background was considered the endpoint.

Cross-reactivity tests. Two hundred μl of rabbit anti-*Aspergillus*, anti-*Penicillium*, or anti-*Stachybotrys* were added to 3 sets of 4 tubes numbered from 1 to 12. Tubes 1, 5, and 9 received 100 μl of saline. Tubes 2, 6, and 10 received 100 μl of 10 mg/ml of *Aspergillus* extract. Tubes 3, 7, and 11 received 100 μl of 10 mg/ml of *Penicillium* extract, and 100 μl of 10 mg/ml of *Stachybotrys* extract was added to tubes 4, 8, and 12. After vortexing, 1 ml of 0.1 *M* PBS (pH 7.2) was added to each tube and mixed. The tubes were then kept for 3 h at 37 °C, after which they were kept overnight at 4 °C, centrifuged at 10,000 g, and the supernatant from each tube was used in the ELISA testing.

Affinity-purified rabbit anti-*Aspergillus, Penicillium*, and *Stachybotrys* were serially diluted on duplicate rows of microtiter plates coated with either of their respective antigens. All other steps were similar to the ELISA described earlier. Percentage binding of each affinity-purified antibody to each of the mold antigens was then calculated.

The author used the following method to determine cross-reactivity between different mold antigens and to ensure degree of rabbit IgG anti-*Aspergillus*, anti-*Penicillium*, or anti-*Stachybotrys* binding only to its respective proteins. Microtiter plates were coated with 1 of the 3 molds and blocked as above. One hundred μl of serum diluent buffer was then added to all wells. Mold extract, starting at 1 mg/ml, was added to the 2nd row and titered down the column in half-log dilution, followed by addition of 100 μl of rabbit anti-*Aspergillus*, anti-*Penicillium*, or anti-*Stachybotrys* to all wells of the particular columns. Following the addition of the enzymes labeled anti-rabbit IgG, incubation, and washing, substrate color development was measured at 405 nm. Results were calculated as percentages of inhibition in antigen-antibody reaction.

Statistical analysis. General Linear Model (GLM) for Windows, version 11.5 (SPSS, Inc., Chicago, IL), with advanced option, was used in this study.

Results

The results of the immunodiffusion, Western blot, and ELISA testing on different dilutions of control and immune rabbit sera against *Aspergillus*, *Penicillium*, and *Stachybotrys* extracts are shown in Table 1. Undiluted control (nonimmunized) rabbit serum resulted in no precipitating bands in the immunodiffusion assay and in 2 bands in the Western blot assay (data not shown). At a dilution of 1:1000 in the ELISA test, the control rabbit serum IgG values against *Penicillium* and *Stachybotrys* were elevated significantly (0.45 and 0.62), but they were less than 0.2 OD against *Aspergillus*. When sera from immunized rabbits were reacted with all 3 mold antigens, only undiluted immunized rabbit sera resulted in an immunodiffusion precipitation banding with *Aspergillus, Penicillium*, or *Stachybotrys* extracts. At a dilution of 1:1000, none of the highly immunized rabbit sera resulted in clear precipitation banding. The use of these antibodies in Western blot and ELISA resulted in a reactivity of a specific antigen with its respective antibody with 10 or more bands (immunoblot) and an OD that exceeded 2.50 (ELISA). However, the most sensitive method for the detection of specific or nonspecific antibodies in rabbit sera was ELISA, because at dilutions of 1:32,000 the immunized rabbit gave an OD that exceeded 0.4 with its respective antigen. As ELISA was the most sensitive method of detection, this author selected it as the most appropriate for the cross-reactivity study.

The IgG antibody was measured by ELISA against different mold antigens in non-immunized (control) rabbit

Table 1.—Reactions of Different Dilutions of Rabbit Sera with *Aspergillus* (A), *Penicillium* (P), and *Stachybotrys* (S) Antigens Using Immunodiffusion (I), Western Blot (W), and Enzyme-Linked Immunosorbent Assay (ELISA) (E) Methods

Antibody	Method	Concentrated			1:1000			1:4000			1:8000			1:16000			1:32000		
		A	P	S	A	P	S	A	P	S	A	P	S	A	P	S	A	P	S
Normal	I	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
rabbit	W	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
serum	E	+	+	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
Rabbit	I	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
anti-	W	++++	++	+	++	+	−	+	−	−	−	−	−	−	−	−	−	−	−
Aspergillus	E	+++++	+	++	+++	−	−	++	−	−	+	−	−	+	−	−	+	−	−
Rabbit	I	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
anti-	W	++	++++	+	+	++	−	−	−	−	−	−	−	−	−	−	−	−	−
Penicillium	E	++++	+++++	+	+	++	+	−	++	−	−	+	−	−	+	−	−	−	−
Rabbit	I	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
anti-	W	++	++	++++	+	+	+++	−	−	+	−	−	−	−	−	−	−	−	−
Stachybotrys	E	++	++	+++++	+	+	+++	−	−	++	−	−	+	−	−	+	−	−	−

Notes: ELISA +, 0.4–0.6; ++, 0.61–0.9; +++, 0.91–1.4; ++++, 1.41–2.0; +++++, 2.1–3.0. Western Blot +, 1–2 bands; ++, 3–4 bands; +++, 4–6 bands; ++++, 6–10 bands; and +++++, >10 bands.

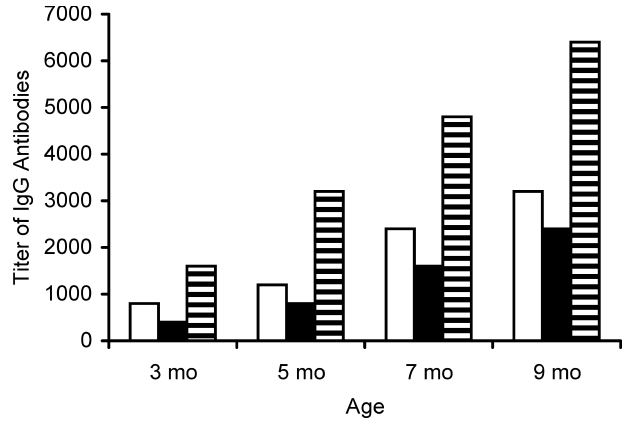

Fig. 1. Titers of IgG antibodies against *Aspergillus niger* (□), *(Penicillium notatum)* (■), and *Stachybotrys chartarum* (▤) in blood samples of nonimmunized rabbits at 3, 5, 7, and 9 mo of age. Ig = immunoglobulin.

Table 2.—Simultaneous Measurement of Rabbit Immunoglobulin G Prepared against *Aspergillus, Penicillium,* and *Stachybotrys* on Plates Coated with *Aspergillus, Penicillium,* or *Stachybotrys* Antigens

Rabbit anti:	Titer of rabbit IgG antibody against: *Aspergillus* extracts	*Penicillium* extracts	*Stachybotrys* extracts
Aspergillus	1:400,000	1:50,000	1:4,800
Percentage binding	100.0	12.5	1.2
Penicillium	1:12,800	1:100,000	1:6,400
Percentage binding	12.8	100.0	6.4
Stachybotrys	1:6,400	1:6,400	1:50,000
Percentage binding	12.8	12.8	100.0

serum at 3, 5, 7, and 9 mo of age (Fig. 1). The data showed that although at 3 mo of age some levels of antibodies against *Penicillium* (1:400), *Aspergillus* (1:800), and *Stachybotrys* (1:600) were detected, these antibody levels increased significantly with the age of the rabbits. The highest level of antibodies was detected against *Stachybotrys*, which increased in the nonimmunized rabbit sera—from 1600 to 3200, 4800, and 6400 at 5 mo, 7 mo, and 9 mo of age, respectively.

The sera of rabbits immunized with each mold were also tested by ELISA for reactivity with other mold antigens (Table 2). Anti-*Aspergillus* serum reacted with the *Aspergillus*-coated microtiter plate gave an IgG titer of 1:400,000, whereas those against *Penicillium*- and *Stachybotrys*-coated wells were 1:50,000 and 1:4,800, respectively. Rabbit anti-*Penicillium* serum produced the following IgG titers when reacted with the mold antigens: 1:100,000 (*Penicillium*), 1:12,800 (*Aspergillus*), and 1:6400 (*Stachybotrys*). Similarly, anti-*Stachybotrys* gave an IgG titer of 1:50,000 against *Stachybotrys* antigens, 1:6400 against *Aspergillus* antigens, and up to a dilution of 1:6400 against *Penicillium* antigens (Table 2). These results suggest the presence of cross-reactivity.

Cross-reactivity of mold antibodies. ODs of rabbit anti-*Aspergillus*, *Penicillium*, or *Stachybotrys* extracts, before and after absorption with mold-specific antigens, and their applications to microtiter plates coated with these mold extracts, are shown in Figures 2–4. In Figure 2 is shown the following: (a) reactivity of rabbit anti-*Aspergillus* with *Aspergillus* extract resulted in an OD of 2.62 in ELISA; (b) *Aspergillus* extract in liquid phase inhibited binding of *Aspergillus* antibodies to *Aspergillus*-coated wells by 70% (from OD of 2.62 to

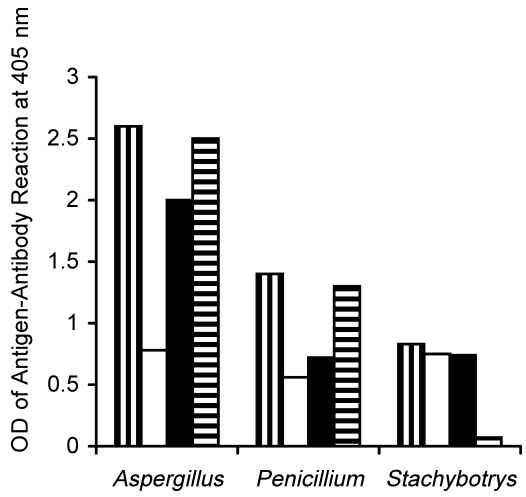

Fig. 2. Percentage of rabbit anti-*Aspergillus* binding to *Aspergillus, Penicillium,* and *Stachybotrys* before (▥) and after absorption with *Aspergillus* (□), *Penicillium* (■), and *Stachybotrys* (▤). OD = optical density.

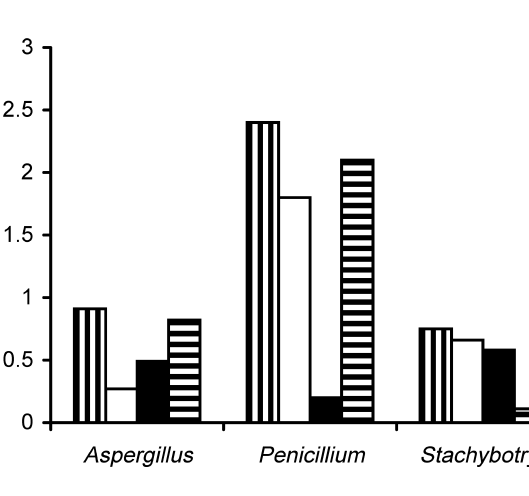

Fig. 3. Percentage of rabbit anti-*Penicillium* binding to *Aspergillus, Penicillium,* and *Stachybotrys* before (▥) and after absorption with *Aspergillus* (□), *Penicillium* (■), and *Stachybotrys* (▤). OD = optical density.

0.78); and (c) the presence of *Penicillium* or *Stachybotrys* in the liquid phase inhibited the binding by 24% and 4%, respectively. The binding of rabbit anti-*Aspergillus* to *Penicillium*-coated wells resulted in an OD of 1.44. In the presence of liquid-phase extracts of *Aspergillus*, *Penicillium*, and *Stachybotrys*, the OD was reduced to 0.55 (inhibition by 61.1%), 0.73 (inhibition by 49.0%), and 1.34 (inhibition by 7.0%), respectively. Conversely, reactivity of rabbit anti-*Aspergillus* with *Stachybotrys* revealed an OD of 0.83, which in the presence of *Aspergillus* and *Penicillium* in the liquid phase was inhibited by 9–10%. The inhibition of rabbit anti-*Aspergillus* binding to *Stachybotrys*

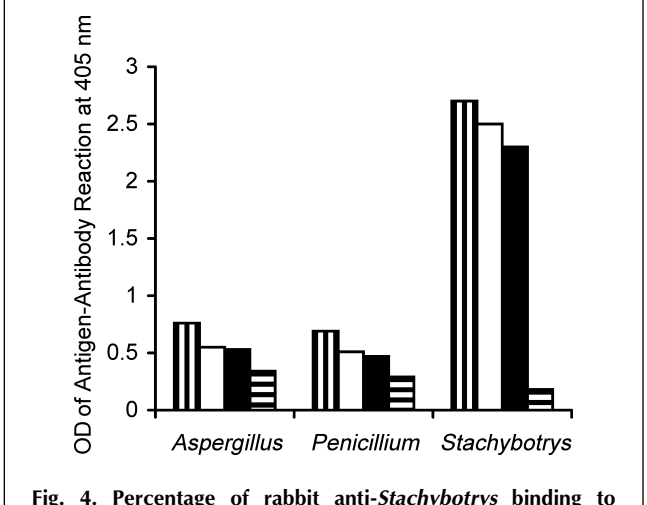

Fig. 4. Percentage of rabbit anti-*Stachybotrys* binding to *Aspergillus, Penicillium,* and *Stachybotrys* before (▥) and after absorption with *Aspergillus* (□), *Penicillium* (■), and Stachybotrys (▤). OD = optical density.

(with an OD of 0.83) by *Stachybotrys* extract in the liquid phase was 92% (i.e., a reduction from 0.83 to 0.07 OD).

The degree of binding of rabbit anti-*Penicillium* to *Aspergillus*-, *Penicillium*-, or *Stachybotrys*-coated wells is shown in Figure 3. Rabbit anti-*Penicillium* binding to *Aspergillus* gave an OD of 0.91. Absorption with *Aspergillus*, *Penicillium*, and *Stachybotrys* extracts inhibited the binding of rabbit anti-*Penicillium* by 71%, 46%, and 10%, respectively. In comparison, the binding of *Penicillium* antibodies to *Penicillium* extract gave an OD of 2.4, which was inhibited by (a) 25% after absorption with *Aspergillus*, (b) 92% after absorption with *Penicillium*, and (c) 12.5% after absorption with *Stachybotrys* extracts. The binding of rabbit anti-*Penicillium* to *Stachybotrys*-coated plates had an OD of 0.75, which was reduced by 12% and 23% after absorption with *Aspergillus* and *Penicillium*, respectively. Following absorption with *Stachybotrys*, the aforementioned reaction was inhibited by 85%.

The binding of rabbit anti-*Stachybotrys* before and after absorption with each mold extract is summarized in Figure 4. The binding of anti-*Stachybotrys* to *Aspergillus*-coated wells gave an OD of 0.76, and absorption with *Aspergillus*, *Penicillium*, and *Stachybotrys* resulted in inhibitions of 28%, 30%, and 45%, respectively. The results for anti-*Stachybotrys* binding to *Penicillium*-coated wells produced similar results to that of the *Aspergillus* assay. The binding of rabbit anti-*Stachybotrys* to *Stachybotrys* antigens resulted in an OD of 2.72. In the presence of *Aspergillus*, and *Penicillium* antigens in the liquid phase, the binding of anti-*Stachybotrys* to *Stachybotrys* extracts was inhibited by approximately 8–15%. In the presence of the *Stachybotrys* extract, the inhibition was 93%.

Table 3.—Reaction of Rabbit Antimold Extracts with Specific and Nonspecific Mold Antigens Expressed by Optical Density at 405 nm and Percentage Inhibition

Mold antigens	Rabbit antimold extracts		
	Aspergillus	*Penicillium*	*Stachybotrys*
Aspergillus	2.62 (100%)	0.91 (38%)	0.76 (28%)
Penicillium	1.44 (54%)	2.43 (100%)	0.69 (25%)
Stachybotrys	0.83 (32%)	0.75 (31%)	2.72 (100%)

The degree of binding calculated from Figures 2–4 is summarized in Table 3. Compared with antimold binding to its respective extracts, results showed that rabbit anti-*Aspergillus* reactivity with *Penicillium* and *Stachybotrys* extracts resulted in 54% and 32% binding, respectively. Anti-*Penicillium* binding to *Aspergillus* and *Stachybotrys* was 38% and 31%, and anti-*Stachybotrys* binding to *Aspergillus* and *Penicillium* was 28% and 25%, respectively.

Cross-reactivity of affinity-purified mold antibodies. Results of affinity-purified mold-specific antibodies and their simultaneous reactivity with *Aspergillus*-, *Penicillium*-, and *Stachybotrys*-coated microtiter wells are shown in Figure 5 and are summarized in Table 4. In the ELISA procedure, affinity-purified rabbit antimold antigens developed until the OD reached at least 1.5 at 405 nm. The ODs for affinity-purified rabbit anti-*Aspergillus* reactivity with *Aspergillus*-, *Penicillium*-, and *Stachybotrys*-coated wells were 1.7, 0.36, and 0.14, respectively. In essence, purified anti-*Aspergillus* reacted with 21% (0.36 OD) of *Penicillium* antigens and with only 8.2% (0.14 OD) of *Stachybotrys* extract. Similarly, affinity-purified anti-*Penicillium* reactivity with *Penicillium* antigens resulted in an OD of 1.58 (100% binding), with *Aspergillus* the antigens resulted in an OD of 0.3 or 19.6% binding, and with *Stachybotrys* the antigens resulted in an OD of 0.11 or 6.9% binding. The reactivity of affinity-purified rabbit anti-*Stachybotrys* with (a) anti-*Stachybotrys* antigens, (b) *Penicillium*, and (c) *Aspergillus*-coated wells gave an OD of 2.30 (100.0%), 0.22 (9.6%), and 0.20 (8.7%) respectively.

The results of the ELISA inhibition analysis for each affinity-purified rabbit antigen to the 3 molds are shown in Figures 6–9. Affinity-purified rabbit anti-*Aspergillus* was mixed with varying dilutions (from 1 mg/ml to 1 μg/ml) of each of the aforementioned mold extracts in the liquid phase and applied to microtiter plated wells coated with *Aspergillus*. *Aspergillus* extract at 1 mg/ml produced 79.4% inhibition of antibody binding to *Aspergillus*-coated wells. The inhibition by *Aspergillus* extract was reversed by lowering the concentration of the antigen in the liquid phase. The binding of anti-

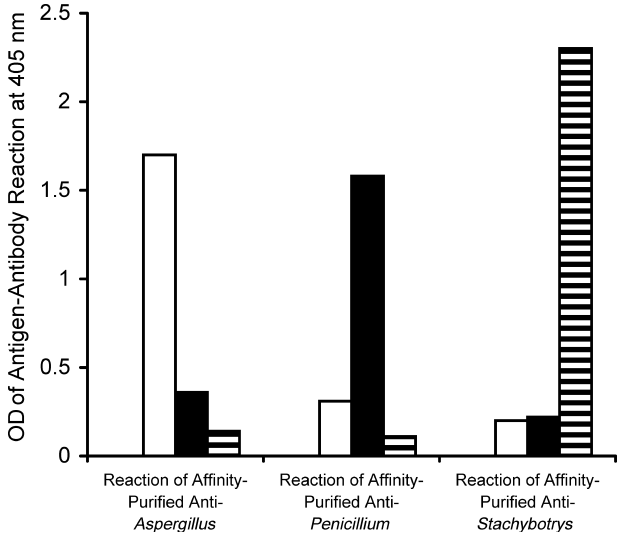

Fig. 5. Reaction of affinity-purified anti-*Aspergillus*, affinity-purified anti-*Penicillium*, and affinity-purified anti *Stachybotrys* with *Aspergillus* (□), *Penicillium* (■), and *Stachybotrys* (▤). OD = optical density.

Table 4.—Reaction of Affinity-Purified Rabbit Antimold Extracts with Specific and Nonspecific Mold Antigens, Expressed by Optical Density at 405 nm and Percentage Inhibition

Mold antigens	Affinity-purified rabbit antimold extracts		
	Aspergillus	*Penicillium*	*Stachybotrys*
Aspergillus	1.71 (100%)	0.31 (19.6%)	0.20 (8.7%)
Penicillium	0.36 (21%)	1.58 (100%)	0.22 (9.6%)
Stachybotrys	0.14 (8.2%)	0.11 (7%)	2.30 (100%)

Aspergillus to *Aspergillus* extract was inhibited by 9.6% with *Penicillium* and by 4.9% with *Stachybotrys* extract (Figs. 6 and 9). The anti-*Penicillium* bound to *Penicillium* antigens was inhibited 79.2% by *Penicillium* extract in liquid phase, by *Aspergillus* (8.9%), and by *Stachybotrys* extract (3.3%) at 1 mg/ml (Figs. 7 and 9). At a concentration of 1 mg/ml, *Stachybotrys* extract in liquid phase caused 95% inhibition in the binding of *Stachybotrys* antibody to *Stachybotrys*-coated wells. *Aspergillus* and *Penicillium* extracts (1 mg/ml) resulted in 12.3% and 9.3% inhibition of *Stachybotrys* antibody binding to *Stachybotrys*-coated wells (Figs. 8 and 9, respectively).

Discussion

In a recent study, Vojdani et al.[33] measured IgA, IgM, and IgG antibodies against *Penicillium notatum*, *Aspergillus niger*, *Stachybotrys chartarum*, and satratoxin H in the blood of 500 healthy blood donor controls, 500 random patients, and 500 patients exposed to

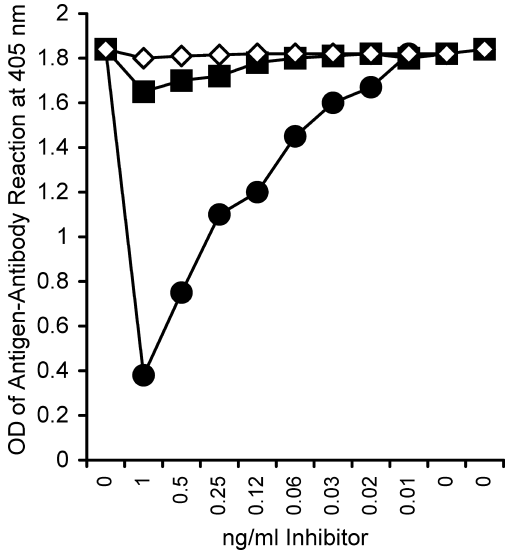

Fig. 6. Anti-*Aspergillus* inhibition with *Aspergillus* (●), *Penicillium* (■), and *Stachybotrys* (◇) in IgG inhibition analysis. OD = optical density.

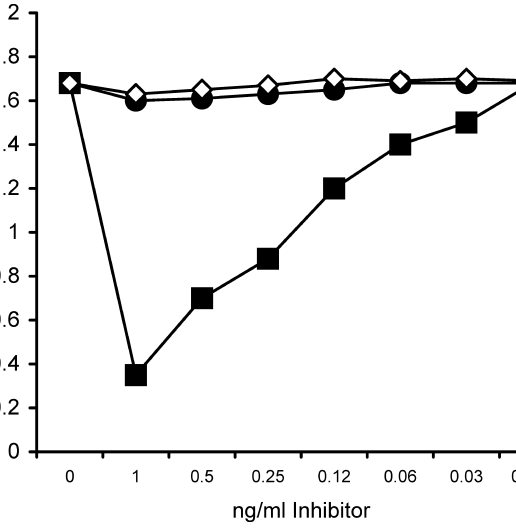

Fig. 7. Anti-*Penicillium* inhibition with *Aspergillus* (●), *Penicillium* (■), and *Stachybotrys* (◇) in IgG inhibition analysis. OD = optical density.

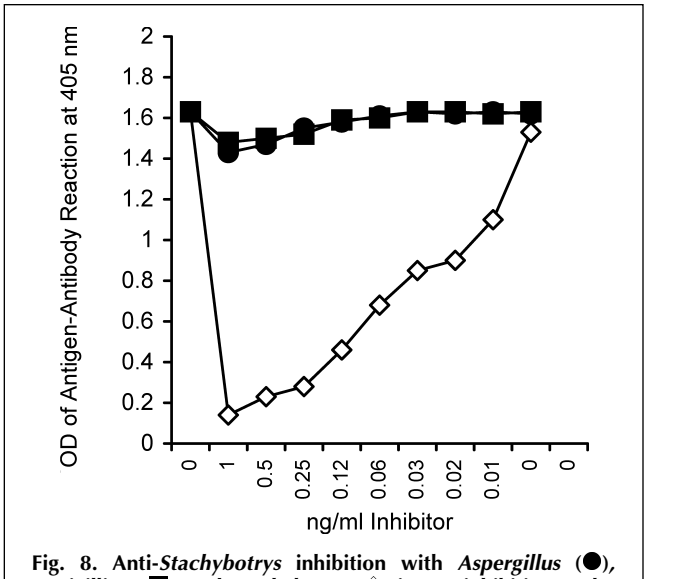

Fig. 8. Anti-*Stachybotrys* inhibition with *Aspergillus* (●), *Penicillium* (■), and *Stachybotrys* (◇) in IgG inhibition analysis. OD = optical density.

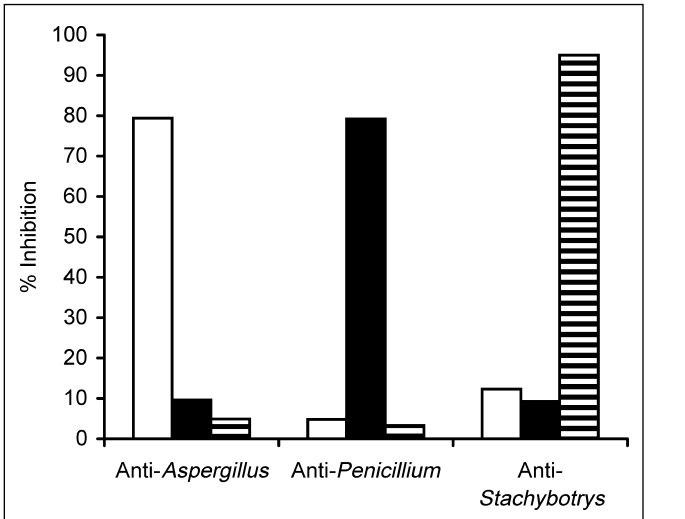

Fig. 9. Inhibition of the binding of the 3 different mold extracts in the solid phase with their antigens by 1 mg/ml of the 3 antigens—*Aspergillus* (□), *Penicillium* (■), and *Stachybotrys* (▤)—in liquid phase.

molds. The antibodies against all 3 mold extracts were significantly greater in the patients than in the controls. It was concluded that detection of antibodies to molds probably resulted from the antigenic stimulation of the immune system and reactivity of antibodies with mold antigens.[33] Those results led the authors to believe that either patients were exposed simultaneously to the 3 molds, or that these antibodies were the result of cross-reactive antigens. Given that antibodies against several mold extracts may be present in human serum, the authors decided to use rabbits that had been immunized with a special preparation of mold extracts to study cross-reactivity between *Aspergillus*, *Penicillium*, and *Stachybotrys*. If exposure to mold is suspected, part of the diagnosis should include the use of very sensitive techniques (e.g., ELISA,[14] RAST[22]) or less-sensitive methods (e.g., immunodiffusion,[13] MAST[38]) for the detection of IgE and IgG antibodies in serum.

In the current study, the author used immunodiffusion, Western blot, and ELISA techniques and concluded that immunodiffusion is not a sensitive technique; therefore, it is not appropriate for cross-reactivity studies. Although in this study the Western blot was very sensitive, it is difficult to perform on many samples, it is a dichotomous test, and titration of cross-reactive antibody levels is impossible. In the current study, fungal antigens were prepared in a manner similar to those

described by others[31–33] and mixed with commercially available allergenic mold in a ratio of 1:1. This preparation enabled examination of mold extracts with a maximum number of antigens in a cross-reactivity study in which ELISA was used.

In previous cross-reactivity studies in which investigators used RAST, immunoblot, or ELISA inhibition, they used either human serum, rabbit serum, or both.[1,13,26–28,31,39–44] Inasmuch as both rabbit and human serums contain specific and nonspecific IgG and IgE, it became imperative for investigators to know whether these antibodies recognize the same or different antigens.[32] For example, human serum with high levels of antibodies against *Stachybotrys* and low levels of antibodies against *Penicillium* may result in many bands in immunoblots of *Stachybotrys* extract and few bands with *Penicillium* extract with exactly the same kD of *Stachybotrys* antigen. This reaction of serum containing low levels of *Penicillium* antibody—but high levels of *Stachybotrys* antibody with *Penicillium* antigen—can convey an incorrect impression about cross-reactivity between these fungi. Moreover, because during SDS[26] PAGE and transblotting[43] the extracts undergo complete denaturation (SDS + mercaptoethanol + boiling), the immune reaction with serum may not represent the original allergenic extracts. Indeed, in a study of a patient who had a positive skin-prick test result to mushroom and 4 types of molds, the immunoblot assay revealed immunoglobulin E antibodies directed against similar molecular-weight proteins in the raw mushroom and *Alternaria tenuis*, *Fusarium vasinfectum*, and *Hormodendrum cladosporioides* extracts. The protein bands in protein electrophoresis were absent in the cooked extracts.[42] As is the case in humans, rabbits can also synthesize some levels of antibodies against environmental molds that result in false-positive reactions.[17,45] This issue was not addressed in earlier cross-reactive studies in which rabbit serum was used.[1,13,14,26,27,29,31,40–44]

Sera from nonimmunized rabbits and from rabbits immunized with specific mold antigens before and after affinity purification were used in our ELISA assays and for demonstration of possible cross-reactivity between *Aspergillus*, *Penicillium*, and *Stachybotrys*. Our results provided evidence that nonimmunized rabbit sera contained significant amounts of antibodies to *Aspergillus*, *Penicillium*, and *Stachybotrys*—which increased with age. Similar observations in rabbits have been reported for other molds and extracellular polysaccharides.[17,45] The use of unpurified rabbit antisera in immunological assays may result in false-positive reactions and false impressions about the degree of cross-reactivity among mold species and genera. Therefore, the use of affinity-purified antibodies or monoclonal antibodies is best for cross-reactivity studies.

Both ELISA inhibition and the use of affinity-purified antibodies confirmed that the degree of cross-reactivity between *Aspergillus* and *Penicillium* is 19–21%. This figure is greater than the cross-reactivity between *Stachybotrys* and *Aspergillus* or *Penicillium*, which was 9–10%. These findings cannot be attributed to interfering factors from the media in which they were grown because, after culture, the mycelia were washed 4 times with buffer, and the rabbit antibody prepared against mold extracts did not react with malt extract broth and cellulose broth in our sensitive ELISA.

Only limited information is available that elucidates the relationship between the cross-reactivity of *Stachybotrys* with other molds. In a preliminary study, the cross-reactivity of the *S. chartarum* antigenic component with 10 other fungal species was identified by the inhibition immunoblotting method.[31] At a concentration of 20 μg/ml, although *S. chartarum* extract in the liquid phase inhibited the binding of the *S. chartarum* antibody to the *S. chartarum* antigen in the solid phase by 100%, inhibition with *Penicillium* and *Aspergillus* was 0%. However, with increased concentrations of *Penicillium* and *Aspergillus* to 200 μg/ml and 2000 μg/ml, the inhibition was 23% and 97% by *Aspergillus*, respectively, and 21% and 93% by *Penicillium*, respectively.[31] The inhibition of *Stachybotrys* antibody to *S. chartarum* antigen under antigen excess in vitro conditions is not realistic because in both the skin-prick test and ELISA the concentration of extracts used did not exceed 20 μg/ml.

In a recent study, Barnes et al.[46] studied IgE-reactive proteins of *S. chartarum* by enzyme-immune assay and reported that 65 of 132 (49.2%) sera tested contained IgG against *S. chartarum* and that 13 of 139 (9.4%) sera tested contained IgE against *S. chartarum*.[46] However, in a letter to the editor,[47] it was indicated that the detected IgG and IgE antibodies against *Stachybotrys* were related to the patients' exposure to other molds and to cross-reactivity to other fungal antigens. This latter assumption was based upon 2 sources[48,49]: (1) an abstract presented at a society meeting describing a study in which affinity-purified serum was not used (unless the full-length article is published, one cannot examine the methodologies and reach any conclusions about the degree of cross-reactivity)[49]; and (2) a position paper by the California Department of Health (posted on the Internet) in which not a single experiment was conducted.[48] I conclude, therefore, that valid studies of *Stachybotrys*, serologies of other molds, and the degree of cross-reactivity among molds require the following: (a) preparation of extracts with the maximum antigenic component for use in its natural form, and (b) use of an affinity-purified human or rabbit serum in a sensitive and quantitative assay (ELISA). On the basis of these criteria, I determined that the cross-reactivity between *Aspergillus* and *Penicillium* was 19.6–21.0%, between *Aspergillus* and *Stachybotrys* was 8.2–8.7%, and between *Aspergillus* and *Stachybotrys* was 7.0–9.6%. The health implications of these findings for patients

exposed to *Stachybotrys* are that, if after exposure, 10,000 units of antibodies against *Stachybotrys* are detected in blood, only 10% or 1000 units of these antibodies are related to cross-reactivity with other molds; the remaining 9,000 units are specific to *Stachybotrys*. Thus, detection of high levels of antibodies against *Stachybotrys* antigens indicates exposure to *Stachybotrys*, and not to *Aspergillus* or *Penicillium*.

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