Antibodies to Molds and Satratoxin in Individuals Exposed in Water-Damaged Buildings

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ABSTRACT. Immunoglobulin (Ig)A, IgM, and IgG antibodies against *Penicillium notatum, Aspergilus niger, Stachybotrys chartarum,* and satratoxin H were determined in the blood of 500 healthy blood donor controls, 500 random patients, and 500 patients with known exposure to molds. The patients were referred to the immunological testing laboratory for health reasons other than mold exposure, or for measurement of mold antibody levels. Levels of IgA, IgM, and IgG antibodies against molds were significantly greater in the patients ($p < 0.001$ for all measurements) than in the controls. However, in mold-exposed patients, levels of these antibodies against satratoxin differed significantly for IgG only ($p < 0.001$), but not for IgM or IgA. These differences in the levels of mold antibodies among the 3 groups were confirmed by calculation of z score and by Scheffé's significant difference tests. A general linear model was applied in the majority of cases, and 3 different subsets were formed, meaning that the healthy control groups were different from the random patients and from the mold-exposed patients. These findings indicated that mold exposure was more common in patients who were referred for immunological evaluation than it was in healthy blood donors. The detection of antibodies to molds and satratoxin H likely resulted from antigenic stimulation of the immune system and the reaction of serum with specially prepared mold antigens. These antigens, which had high protein content, were developed in this laboratory and used in the enzyme-linked immunosorbent assay (ELISA) procedure. The authors concluded that the antibodies studied are specific to mold antigens and mycotoxins, and therefore could be useful in epidemiological and other studies of humans exposed to molds and mycotoxins.
<Key words: antibody, *Aspergillus*, ELISA, molds, mycotoxins, *Penicillium*, *Stachybotrys*>

WATER INTRUSION into houses and office buildings leads to the amplification of molds and bacteria.[1–6] These organisms are known to produce toxic byproducts that include endotoxins (lipopolysaccharides), β-D-glucans, and mycotoxins (e.g., trichothecenes, ochratoxins, and aflatoxins), as well as solvents. These compounds have been demonstrated in water-damaged buildings and from artificially contaminated building

materials.[7–11] Molds can be aerosolized, resulting in indoor air contaminated with spores and hyphae fragments.[11] In addition, mycotoxins have been identified in ventilation duct dust and in the air in buildings in which occupants have experienced adverse health effects related to mold exposure.[12–18]

Exposure to airborne molds can result in immunologic-mediated illnesses, as well as pathological and inflammatory processes. Immunologic health effects include upper and lower respiratory allergy, asthma, and hypersensitivity pneumonitis.[19–25] Immunoglobulin (Ig)E antibodies to a variety of molds are demonstrable in individuals with suspected allergic sensitivity to molds.[26–29] By using *Stachybotrys* extracts in enzyme-linked immunosorbent assay (ELISA) and radioallergosorbent (RAST) tests, researchers have measured mold-specific IgG and IgE in the blood of individuals exposed to toxigenic fungi.[5] These investigators found no elevated levels of IgG or IgM antibodies to *Stachybotrys chartarum* that had a statistically significant association with health outcome.[5] In a mouse study, an increase in IgA production and IgA nephropathy were reported after injection of trichothecene or vomitoxin.[19] Similar findings were reported when IgE, IgG, and IgA antibodies against *S. chartarum* were identified in patients with asthmatic or mycotoxicosis symptoms.[30] Mycelium extract was used in the ELISA assay, and the *Stachybotrys*-specific IgG and IgA were detected in the group of exposed subjects, but not in control groups. IgA levels were significantly higher ($p < 0.01$) and IgG levels less high ($p < 0.05$) in the patient group than in the control groups. IgE levels did not differ between groups. It has been suggested that exposure to *Stachybotrys* does not cause IgE-mediated allergy in humans and that the IgA response better reflects exposure to the fungus than does the IgG response.[30]

Other investigators have reported that serum IgA level was a more specific factor than IgG level for indicating Farmer's Lung, a disease associated with fungal exposure. They reached the same conclusion concerning antibodies against *Aspergillus fumigatus*.[31,32] When specific IgA was detected in patients' sera, the IgA concentrations in bronchoalveolar lavage were high. This suggests that the concentration of serum IgA may depend on the magnitude of respiratory exposure to fungi.[33] In contrast, IgG antibodies to 8 different molds in subjects with Farmer's Lung disease, compared with healthy farmer controls, were significantly elevated above their healthy counterparts. The Farmer's Lung group had positive titers against several species of mold, whereas the control farmers usually had a positive titer against 1 or 2 microbes. A positive association between IgG antibody levels and chronic bronchitis and diffusing capacity was observed in the Farmer's Lung patients, but not in the control farmers.[34]

Pathological and inflammatory conditions are often caused by molds. Molds and yeast invade tissues in both immunocompromised and normal individuals, establishing an infectious state that is difficult to manage clinically.[35–50] With respect to human infants, *Stachybotrys chartarum* has been associated with pulmonary hemosiderosis.[51–54] The organism *S. chartarum*, isolated from the lungs of an infant, produced a hemolysin (stachylysin), siderophore, and stachyrase A. Apparently, different strains of *S. chartarum* produce different quantities and types of various highly toxic materials, including trichothecenes.[55–61] In an earthworm model, stachylysin increased the permeability of blood vessels, causing leakage through the blood vessel endothelium and walls.[59] Additionally, pathology may result from interference with lung surfactant synthesis by *S. chartarum* spores and isosatratoxin-F in juvenile mice.[62] Ultrastructural changes in Type II alveolar cells (condensed mitochondria, increased cytoplasmic rarefaction, and distended lamellar bodies with irregular shapes) have been observed following exposure to *S. chartarum*.[62–65] Thus, hemolysins, siderophores, and proteases appear to have an important role in the pathogenesis of mold infections.[55–69]

Numerous species of molds, including some found indoors in contaminated buildings, produce mycotoxins.[11,69,70] Controlled laboratory exposure of animals to mycotoxins has pointed to several potential toxic effects on humans (e.g. cytotoxicity, immunosuppression, mitochondrial toxicity, carcinogenicity, nephrotoxicity, and deoxyribonucleic acid [DNA] adducts).[71–87] The adverse health effects from exposure to a mixture of molds and their metabolites are different from those related to a single mycotoxin under controlled conditions. Thus, humans express symptoms that include the central and peripheral nervous systems; neurocognitive deficiencies; problems with skin, upper and lower respiratory tracts, gastrointestinal tract, and skeletal-muscular system; chronic fatigue; and flu-like illnesses.[5,17,23,88–94]

Inasmuch as the respiratory tract is the major route of human exposure to fungi and mycotoxins in water-damaged buildings, we conducted this study to measure specific IgG, IgM, and IgA antibodies to 3 mold species commonly found in such environments. An ELISA procedure to detect antibodies to satratoxin H was also undertaken. Simultaneous detection of antibodies against toxigenic molds and their mycotoxins is considered a biomarker of exposure to molds and their metabolites, and may explain clinical disease processes resulting from mold exposure in the workplace and other environments.[93–95]

Materials and Method

Study subjects. The study population consisted of 500 patients exposed to molds in water-damaged build-

ings, from 3 different states—143 from California (64 males, 79 females); 159 from Arizona (72 males, 87 females), and 198 from Texas (83 males, 115 females)—who were referred to Immunosciences Lab, Inc. (Beverly Hills, California) by 3 of the authors (MRG, GH, AWC). Patients' ages ranged from 22 to 76 yr. Environmental engineering firms tested approximately 60 damaged buildings by swab samples, tape transfer, and viable microbial activities and reported *Aspergillus*, *Penicillium*, and *Stachybotrys* at a level of > 2,000 colony-forming units per m^3. Patients had lived in these buildings for periods ranging from several weeks to more than 2 yr and had reported unpleasant musty odors to their referring physicians.

The patients had the following symptoms in common: neurological and behavioral symptoms (memory loss, blurred vision, migraine, loss of balance, cognitive deficit), fatigue, nausea, rhinitis, sinusitis, rashes, and allergies. Blood samples were drawn from the patients and sent to Immunosciences Lab (Beverly Hills, California) to test for antibodies against the 3 most common molds detected in their respective buildings. Antibodies to satratoxin H were also determined. For comparison, we used 500 blood samples obtained from healthy, asymptomatic blood donors from California of similar age and sex. An additional 500 individuals, whose blood had been sent to the laboratory for health reasons other than mold exposure, were selected randomly from our patient population. Blood samples from the mold-exposed patients, randomly selected patients, and controls were tested with ELISA for levels of IgG, IgM, and IgA against *Aspergillus niger, Pencillium notatum,* and *Stachybotrys chartarum,* and for satratoxin H antigens.

Preparation of fungal antigen. The protocol we followed for preparation of optimal fungal antigen extracts was based on procedures reported previously.[96–99] Molds (*Stachybotrys chartarum, Penicillium notatum,* and *Aspergillus niger*) were obtained from the American Type Culture Collection (Rockville, Maryland). The molds were first cultured in 2% malt extract agar for 8 days at 25 °C, after which spore suspensions were prepared in 0.1 *M* phosphate-buffered saline (PBS) pH 7.4 containing 0.05% Tween 20 (Sigma Aldrich [St. Louis, Missouri]). One milliliter of each spore suspension was inoculated into 100 ml of 2% malt extract broth (for *Stachybotrys*) or cellulose broth (for the other molds) in glass bottles; the cultures were incubated for 10 days at 25 °C. The mycelium was separated from the broth by centrifugation at 2,000 *g* for 20 min, dried in a vacuum dryer, and stored at –70 °C. Dried mycelium containing spores was suspended at 50 mg/ml in 0.1 *M* PBS pH 7.5 containing 0.02% phenylmethylsulfonyl flouride (PSF) and 0.02% sodium azide. Mycelium suspension was sonicated for 5 sec at an output of 70% using a Virsonic 50 cell disruptor (Virtis Co. [Gardiner, New York]). The sonication step was repeated 10 times for maximum cell disruption, and thereafter the suspension was kept on a shaker for 24 hr at 4 °C. After centrifugation at 4,000 *g*, the supernatant was dialyzed at molecular cut-off 2kD against PBS at 4 °C for 24 hr, 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 0.01 *M* PBS, the protein content was determined,[28] and the sample's components were analyzed by 15% sodium dodecyl sulfate (SDS) gel electrophoresis. The protein content of our preparations was compared with allergenic extracts for the same molds obtained from Antigen Laboratories, Inc. (Liberty, Missouri). Many more protein bands were present in our preparations than in the commercial antigens; however, a few bands were present in the allergenic extracts but not in our preparations. Consequently, we mixed our antigenic preparations with the Antigen Laboratories extracts, in a ratio of 1:1, and used the combined extracts in our ELISA assays.

Preparation of satratoxin H. Satratoxin H was prepared in accordance with the method of Johanning et al.,[5] with modification in our laboratory. One hundred mg of dried mycelium was extracted with 2 ml of 20% methanol in chloroform at 40 °C, with repeated sonication for 30 min. The extract was passed through a silica gel Column Whatman LPS-1 (Whatman [Clifton, New Jersey]) and washed with 10 ml of 8% methanol in dichloromethane. The eluent was evaporated under a stream of dry nitrogen, and the remaining oily material was dissolved in 1 ml of ethanol and analyzed by reversed-phase high-performance liquid chromatography with a Model 5600 CoulArray Detector with solvent delivery pump Model 580 and an analytical cell that makes use of the 2 porous graphite electrodes (ESA, Inc. [Chelmsford, Massachusetts]). The column was C-18 rainin, 5 µm, 4.6 × 250 mm, with a 15-min gradient of 60–75% methanol in water, flow of 1 ml/min, and monitoring at 260 nm. Two peaks—one at 10.6 and the other at 12.2 min, corresponding in retention time to satratoxin H and other trichothecenes—were obtained. The total satratoxin H and trichothecene obtained in 100 mg of sample were estimated to be about 1.5 µg and 1.7 µg, respectively. Satratoxin H was also obtained from the Department of Microbiology, Texas Tech University, Health Sciences Center (Lubbock, Texas).

Binding of satratoxin to human serum albumin (HSA). Satratoxin was coupled to the carrier protein-HSA with 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide-metho-4-toluolsufonate (CCMT) and succinic anhydride. One hundred µg of satratoxin in 100 µl pyridine was reacted with 2 mg of succinic anhydride in a 45 °C water bath with off-and-on vortexing. The reaction mixture was then evaporated to dryness. The satratoxin-hemisuccinate was dissolved in 200 µl of dimethyl formamide and added dropwise to a 1 ml solution of 0.1

M carbonate buffer pH 9.5 containing 10 mg of HSA and 2 mg of CCMT. The mixture was kept on the stirrer for 4 hr, followed by the addition of 1 mg CCMT and adjustment of the pH to 7.5 using 1 *M* hydrochloric acid, followed by an additional incubation of 4 hr. Finally, after the addition of another 1 mg of CCMT, the pH was adjusted to 5.5 and the mixture was kept on the stirrer for 4 hr at room temperature. Uncoupled residues of the reagents and derivatives were removed by dialysis at a cutoff of 2,000 against 0.1 *M* PBS pH 7.2 for 48 hr. After centrifugation at 10,000 *g*, binding of satratoxin to HSA was examined by SDS gel electrophoresis. A shift in the location of the HSA band after the addition of mycotoxin was used as evidence for the binding capacity of satratoxin to the carrier protein.

ELISA for detection of IgG, IgM, and IgA against fungal antigens and mycotoxins. The levels of IgG, IgM, and IgA antibodies against antigens of molds in human sera were analyzed by indirect ELISA. Microtiter plates were coated with 0.1 ml of either HSA (in duplicate to serve as controls) or mold extract and satratoxin at a protein concentration of 10 μg/ml. After incubation, washing, and blocking with 2% BSA blocking solution, 0.1 ml of human serum, at optimal dilution of 1:2 for IgE and 1:100 in serum diluent buffer (2% BSA in 0.1 ml PBS plus 0.01% Tween 20) for IgG, IgM, and IgA, were added into the quadruplicate wells of the plates. Plates were incubated at 37 °C for 2 hr and washed 3 times with PBS Tween 20. Then, 0.1 ml of affinity-purified goat anti-human IgG(γ), IgM(μ), or IgA(α) or light chain specific, conjugated with alkaline phosphatase at dilutions of 1:500, were added and incubated at 37 °C for 1 hr. Color development was measured after repeated washing and the addition of 0.1 ml of paranitrophenylphosphate substrate, incubation for 30 min, and stop solution. The intensity of color was measured spectro-photometrically at 405 nm. For each specimen, the ELISA background readings of wells coated with HSA were automatically subtracted from the ELISA readings of the wells coated with mold antigens. The background readings of wells coated with nonspecific antigen (HSA) reacted with the serum and all other reagents and was less than 12% of the absorbencies of the wells coated with mold antigens. Serially diluted sera from rabbits immunized with molds antigens, and from patients following immunotherapy, with assigned values of 400, 3,200, and 12,800 ELISA units against different molds, were used to construct a standard curve. To determine the ELISA values for unknowns, we plotted the mean absorbances obtained from duplicate wells from each calibrator against the antibody concentration, with the absorbance on the vertical axis and concentration on the horizontal axis.

Coefficients of intra-assay and inter-assay variations and optimal dilution of serum for mold and mycotoxin antibodies. Coefficients of intra-assay variations were calculated by running 5 samples 8 times in 1 assay. Coefficients of inter-assay variations were determined by measuring the same samples in 6 consecutive assays. This replicate testing established the validity of the ELISA assays, determined the appropriate dilution with minimal background, and detected serum IgG, IgM, and IgA against different antigens. Two sera from healthy controls and 2 from patients exposed to molds were used to construct control curves.

The optimal dilutions of sera were determined by diluting sera from 20 different controls, and patients sera, 1:25–1:400 in serum diluent buffer and adding them to duplicate wells coated with either HSA or mold antigens. Dilutions between 1:50 and 1:200 resulted in a good linearity; therefore, a serum dilution of 1:100 was used for all IgG, IgM, and IgA antibody assays.

Antibody-specificity testing by absorption of sera. Specificity of the ELISA assay for molds and satratoxin antibodies was confirmed by mold antigens competition. For this, 3 different sera with high levels of IgG, IgM, and IgA antibodies (optical density in ELISA > 0.8) against *Stachybotrys* were used in different test tubes. One ml of each serum was pre-incubated with 1 mg of either HSA, *Stachybotrys*, *Aspergillus*, *Penicillium*, or *Alternaria* antigens. After mixing, the tubes were kept for 1 hr in a 37 °C water bath, followed by 1 hr of incubation at 4 °C and centrifugation at 3,000 *g* for 10 min. The supernatant was used for measurement of IgG, IgM, and IgA antibody levels against *Stachybotrys* antigens. ELISA values of unabsorbed serum were compared with those for serum absorbed with HSA or with fungal antigens.

Statistical analysis. The main objective of our data analysis was to examine the differences between the levels of IgG, IgM, and IgA antibodies against different molds and a mycotoxin among 3 groups: (1) controls, (2) random patients, and (3) mold-exposed patients. The IgG, IgM, and IgA were considered as 3 dependent variables with 1 factor variable that divided our samples into the 3 groups mentioned above.

The General Linear Model (GLM) for Windows, version 11.5 (SPSS, Inc. [Chicago, Illinois]), with advanced option, was used in this study. The *z* scores and *p* values were obtained using Hotelling's Trace. In addition, for the post hoc tests, Scheffé's significant difference test was performed on 500 samples in each group. The GLM multivariate procedure provided analysis of variance for multiple dependent variables by 1 or more factor variables.

Results

Fungal contamination of buildings. We were in continuous personal contact with our patients' referring physicians.[93–95,100] Each of the suspect buildings was tested by a different independent environmental

firm for the presence of mold and mold spores. All buildings were contaminated with a variety of mold genera. Inasmuch as *Aspergillus* sp., *Pencillium* sp., and *Stachybotrys chartarum* were the most frequently identified molds, we selected *Aspergillus niger, Penicillium notatum,* and *Stachybotrys chartarum* as representative organisms. Satratoxin H was incorporated in our study as being representative of trichothecene because this mycotoxin is known to be produced by *S. chartarum.*

Detection of IgG, IgM, and IgA antibodies against molds and satratoxin H. Sera from controls, mold-exposed patients, and randomly selected patients were analyzed for the presence of IgG, IgM, and IgA antibodies to the 3 molds and satratoxin H. The mean titers ± standard deviations (*SD*s), as determined by ELISA for each isotype, along with *z* scores and *p* values, are given in Tables 1–4. The serum dilution used was 1:100, which was determined previously to be optimum.[100] IgG, IgM, and IgA titers against *P. notatum, A. niger,* and *S. chartarum* are presented in Tables 1–3, respectively. The *z* scores for all 3 isotypes against *Penicillium, Aspergillus,* and *Stachybotrys* were higher than 3.3. IgG, IgM, and IgA antibodies against satratoxin H are given in Table 4. For satratoxin IgG, the differences between the controls and the molds-exposed group were statistically significant ($p < 0.001$; $z = 11.3$).

To examine statistical differences among the 3 groups for the levels of molds and mycotoxins, the post hoc tests (Scheffé's significant difference tests) were performed and were classified under 3 different subsets, as presented in Table 5. Means for groups in homogenous subsets are based on Type III sum of squares (sample size = 1,500, with 500 subsets in each group). Means that are reported in the same subset are statistically similar. For example, means for IgA (satratoxin) for controls, random patients, and mold-exposed patients are 627, 784, and 759, respectively, which are statistically alike. Similarly, the means for *Aspergillus* and *Stachybotrys* IgA in controls and in random patients are classified under the same subset, whereas the means for IgA in mold-exposed groups are statistically different for both controls and random patients. For all other determinations, 3 different subsets are formed, meaning that IgG and IgM against *Penicillium, Aspergillus, Stachybotrys,* and satratoxin are statistically different in controls, random patients, and mold-exposed patients. For IgA against *Penicillium*—although formation of 3 subsets indicates statistical differences among the 3 groups—the IgA values for *Aspergillus* and *Stachybotrys* were reported in 2 subsets, meaning that the mold-exposed group was statistically different from both controls and random patients. And, finally, satratoxin IgA means are classified under the same subset, indicating that no dif-

Table 1.—Antibody Levels (in ELISA Units) in Response to *Penicillium notatum* in Controls, Randomly Selected Patients, and Mold-Exposed Patients, with *z* Scores and *p* Values

	Controls vs. mold-exposed						Random vs. mold-exposed					
	Controls (n = 500)		Exposed (n = 500)				Controls (n = 500)		Exposed (n = 500)			
Antibody	$\bar{x}$	*SD*	$\bar{x}$	*SD*	*z*	*p*	$\bar{x}$	*SD*	$\bar{x}$	*SD*	*z*	*p*
IgG	620	535	2,159	2,458	13.7	< 0.001	1,383	1,839	2,159	2,458	5.6	< 0.001
IgM	692	551	1,692	2,442	8.9	< 0.001	1,241	1,530	1,692	2,442	3.5	< 0.001
IgA	640	572	1,256	2,163	6.1	< 0.001	853	1,070	1,256	2,163	3.7	< 0.001

Notes: $\bar{x}$ = mean, *SD* = standard deviation, and Ig = immunoglobulin.

Table 2.—Antibody Levels (in ELISA Units) in Response to *Aspergillus niger* in Controls, Randomly Selected Patients, and Mold-Exposed Patients, with *z* Scores and *p* Values

	Controls vs. mold-exposed						Random vs. mold-exposed					
	Controls (n = 500)		Exposed (n = 500)				Controls (n = 500)		Exposed (n = 500)			
Antibody	$\bar{x}$	*SD*	$\bar{x}$	*SD*	*z*	*p*	$\bar{x}$	*SD*	$\bar{x}$	*SD*	*z*	*p*
IgG	618	426	1,795	2,316	11.1	< 0.001	1,349	1,417	1,795	2,316	3.7	< 0.001
IgM	782	420	1,725	2,449	8.5	< 0.001	1,177	1,302	1,725	2,449	4.4	< 0.001
IgA	732	595	1,346	2,456	5.4	< 0.001	849	938	1,346	2,456	4.2	< 0.001

Notes: $\bar{x}$ = mean, *SD* = standard deviation, and Ig = immunoglobulin.

Table 3.—Antibody Levels (in ELISA Units) in Response to *Stachybotrys chartarum* in Controls, Randomly Selected Patients, and Mold-Exposed Patients, with *z* Scores and *p* Values

	Controls vs. mold-exposed						Random vs. mold-exposed					
	Controls (n = 500)		Exposed (n = 500)				Controls (n = 500)		Exposed (n = 500)			
Antibody	$\bar{x}$	*SD*	$\bar{x}$	*SD*	*z*	*p*	$\bar{x}$	*SD*	$\bar{x}$	*SD*	*z*	*p*
IgG	803	530	2,304	2,432	13.5	< 0.001	973	1,234	2,304	2,432	10.9	< 0.001
IgM	629	602	1,940	2,478	11.5	< 0.001	1,115	1,212	1,940	2,478	6.7	< 0.001
IgA	665	665	1,511	2,660	6.9	< 0.001	760	1,086	1,511	2,660	5.8	< 0.001

Notes: $\bar{x}$ = mean, *SD* = standard deviation, and Ig = immunoglobulin.

Table 4.—Antibody Levels (in ELISA Units) in Response to Satratoxin in Controls, Randomly Selected Patients, and Mold-Exposed Patients, with *z* Scores and *p* Values

	Controls vs. mold-exposed						Random vs. mold-exposed					
	Controls (n = 500)		Exposed (n = 500)				Controls (n = 500)		Exposed (n = 500)			
Antibody	$\bar{x}$	*SD*	$\bar{x}$	*SD*	*z*	*p*	$\bar{x}$	*SD*	$\bar{x}$	*SD*	*z*	*p*
IgG	767	641	1,523	1,352	11.3	< 0.001	1,054	1,147	1,523	1,352	5.90	< 0.001
IgM	611	648	1,320	1,590	9.2	< 0.001	1,160	1,170	1,320	1,590	1.80	< 0.060
IgA	715	588	705	868	2.1	< 0.440	747	819	705	868	0.78	< 0.430

Notes: $\bar{x}$ = mean, *SD* = standard deviation, and Ig = immunoglobulin.

ferences were detected among the 3 groups. The statistical differences among the 3 groups for the levels of IgG, IgM, and IgA against molds and mycotoxins were further confirmed by the calculation of exact *z* scores and *p* values, which are provided in Tables 1–4. We considered *p* values < 0.05 and *z* scores > 3.3 to be statistically significant.

Specificity and intra-assay and inter-assay precision. Specificity, and intra-assay and inter-assay precision, for each of the molds, and for several other genera and satratoxin H, have been determined previously.[100] In brief, the coefficients of intra-assay variation calculated for 8 replicates of ELISA assays were 5.7–10.2% for IgG, 5.8–9.2% for IgM, and 5.6–11.3% for IgA. Inter-assay precision was calculated for the same 5 samples assayed in 6 different runs. The inter-assay variations were 7.8–12.7%, 9.5–15.5%, and 10.6–15.3% for IgG, IgM, and IgA, respectively.

Absorption of *Stachybotrys* antibodies with HSA and mold antigens. Similar to our earlier study,[100] 3 different sera with high levels of IgG, IgM, and IgA against *Stachybotrys* were absorbed with nonspecific and specific antigens. Data summarized in Table 6 show that nonspecific proteins, such as HSA, did not change IgG, IgM, and IgA antibody levels against *Stachybotrys*, whereas *Stachybotrys* antigens absorbed the IgG antibody titer levels from 42.7–58%, IgM antibody levels from 21.4–38.5%, and IgA levels from 26.8–34.6%. Similar to IgG, but to a lesser degree, IgM and IgA antibodies were absorbed with *Stachybotrys* antigens. This significant absorption and inhibition of IgG, IgM, and IgA antibodies by fungal antigens is excellent evidence for the specificity of fungal antibodies. Furthermore, other molds' antigens, such as *Alternaria*, were not capable of absorbing levels of *Stachybotrys* antibodies, whereas *Penicillium* and *Aspergillus* absorbed only IgG antibody against *Stachybotrys* from 14–20%. This absorption of anti-*Stachybotrys* antibody by *Penicillium* and *Aspergillus* indicates minor antigenic cross-reactivity between these molds.

Discussion

Adverse health effects from exposure to molds in water-damaged buildings can result in allergic reactions, asthma, hypersensitivity pneumonitis, pulmonary infections, and mucous membrane irritation and toxicity. However, despite this variety of adverse health effects, significant emphasis has been placed mainly on Type I allergy and asthma,[20,21,101–103] and not on the other immunopathologic mechanisms involved in the pathogenesis of Types II–IV allergy.[104]

Hundreds of molds, with thousands of antigens, can contaminate indoor air. In addition, some of these

Table 5.—Multiple Comparisons and Means for Groups in Homogenous Subsets Determined with Scheffé's Post Hoc Tests

Mold/mycotoxin and group	IgG subset			IgM subset			IgA subset		
	1	2	3	1	2	3	1	2	3
Penicillium									
Controls	689			748			679		
Random patients		1,547			1,386			944	
Mold-exposed			2,386			1,985			1,841
Aspergillus									
Controls	578			679			688		
Random patients		1,398			1,214		842		
Mold-exposed			2,035			2,147			1,756
Stachybotrys									
Controls	744			617			668		
Random patients		1,083			1,184		815		
Mold-eposed			2,398			2,086			2,153
Satratoxin									
Controls	725			636			627		
Random patients		1,109			1,163		784		
Mold-exposed			1,460		1,405		759		

Notes: Statistical analyses for examination of differences between the levels of immunoglobulin (Ig)G, IgM, and IgA antibodies against molds and a mycotoxin among 3 different groups using the General Linear Model for Windows, version 11.5 (SPSS, Inc. [Chicago, Illinois]). Means for classification of groups in different subsets were based on the sum of squares of sample size of 500 subjects in each of 3 groups. Means reported in the same subset are statistically similar; if they are classified under a different subset it means that controls differed from random patients, as well as from mold-exposed patients.

Table 6.—Optical Densities of Sera with High Levels of Immunoglobulin (Ig)G, IgM, and IgA Antibodies against *Stachybotrys*, before and after Absorption with Nonspecific and Specific Antigens

Absorption status	Sample 1			Sample 2			Sample 3		
	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
Before absorption									
Optical density	1.54	1.92	1.36	1.10	2.15	1.83	2.30	0.96	0.87
After absorption with:									
Human serum albumin									
Optical density	1.49	1.83	1.27	0.98	2.26	1.95	2.16	0.93	0.91
% inhibition	NS	NS	NS	NS	NS	NS	NS	NS	NS
Stachybotrys chartarum antigens									
Optical density	0.69	1.18	0.89	0.63	1.69	1.34	0.96	0.61	0.57
% inhibition	55.20	38.50	34.60	42.70	21.40	26.80	58.20	36.40	34.50
Aspergillus niger antigens									
Optical density	1.31	1.73	1.27	0.95	1.98	1.76	1.89	0.88	0.78
% inhibition	15.00	10.00	6.60	13.60	8.00	4.00	18.00	8.40	10.30
Penicillium notatum antigens									
Optical density	1.29	1.80	1.26	0.91	1.89	1.81	1.83	0.92	0.79
% inhibition	16.20	6.30	7.30	17.20	12.00	10.90	20.40	4.20	9.20
Alternaria alternata antigens									
Optical density	1.51	1.83	1.29	1.16	2.10	1.72	2.25	0.94	0.80
% inhibition	NS	NS	NS	NS	NS	NS	NS	NS	NS

Note: NS = Nonsignificant.

molds produce potentially toxic metabolites (e.g., mycotoxins and solvents)[3–17,69,70] and can invade tissues (causing aspergilliosis, cryptococcosis, coccidioidomycosis, or pulmonary hemosiderosis)[35–50] by producing proteases, hemolysins, and siderophores.[51–59,61,66–68] Moreover, the potential cytotoxic action of molds and their metabolites includes DNA adducts,[89,82,85,86] adverse effects on pulmonary surfactant synthesis in rodents,[62–65] mitochondrial toxicity,[77,78,83] apoptosis,[73–76] abnormalities of the human immune system,[93] neuro-

cognitive deficits with changes in electroencephalogram,[89,90,94] peripheral neuropathy,[90] autoimmunity,[93,95] and carcinogenesis.[79,81,87] Because it is becoming increasingly apparent that both atopic and nonatopic individuals experience adverse health reactions to mold exposure unrelated to IgE-mediated sensitivity,[89,90,93–95] biomarkers and clinical tests involving the immune system, the respiratory tree, and the central nervous system must be developed and implemented.[89,93–95,100]

Our data clearly show that exposure to molds in water-damaged buildings leads to the production of IgA, IgM, and IgG antibodies to antigens of *Aspergillus niger, Penicillium notatum, Stachybotrys chartarum,* and satratoxin H in subpopulations of patients, and corroborates previous observations on these and other molds by some of the authors.[100] Previous reports on IgG antibodies against *S. chartarum* and *Aspergillus* have produced equivocal results. In one study,[5] 4 of 48 individuals possibly exposed to *S. chartarum* had IgG antibodies to the organism; other researchers found statistically nonsignificant differences between exposed and control groups.[23,105] On the other hand, a recent cross-sectional comparison of water-damaged or mold-contaminated homes in Finland found fungal-specific IgG concentrations in the sera of patients living in houses both with and without mold, with other cases showing a tendency to exhibit higher antibody levels to most fungi than seen in the control groups.[106]

The differences between our study and others in regard to antibodies to molds might be accounted for by differences in the selection of controls and in the preparation of mold antigens. First, in addition to the controls and patients presented herein, we have tested for antibodies to molds in 500 randomly selected individuals with diagnoses other than mold exposure. The titers to molds IgG, IgM, and IgA, and satratoxin H IgA were intermediate to the controls and patients in this study, but significantly different than both the controls and patients presented herein (Tables 1–5). Some of the controls—who represented a healthy population—probably were exposed to molds. Such exposure likely would result in less statistical significance when the control group is compared with the patients. Therefore, if supposedly true controls (individuals not exposed to molds) could be used in this study, the mean ± *SD* would have been less (estimated at 10–20% lower) than reported in Tables 1–5. We anticipate that this would result in higher *z* scores and lower *p* values, or a greater statistical difference between controls and exposed groups. Moreover, in the selection of controls, one assumes they have a likelihood of exposure. Thus, it is apparent from these observations that selection of controls is critical and that it is probable that clinicians miss molds as a possible cause of the illness for which medical attention is being sought.

The second explanation for the differences between the observations presented herein and those of other investigators likely lies in the preparation of antigens. We prepared our fungal antigens similar to the methods described by others,[5,30] but with some modification.[100] Similar culture and suspension techniques were used, but were modified with repeated sonications during 24 hr of extractions, which resulted in higher yield of fungal antigens in suspension. Comparison of commercially prepared fungal antigens with our preparations showed differences in protein concentrations before and after sonications. Protein concentrations of commercial antigens ranged from 0.4 to 2.1 mg/ml, whereas the protein concentrations used in this study ranged from 0.8 to 3.5 mg/ml. After 10 sonications, our protein concentrations ranged from 5.8 to 16.5 mg/ml. In addition, SDS gel electrophoresis on an equal amount of protein (1 mg) revealed (depending on mold species) 9–22 bands in commercial antigens, 7–16 bands in our preparations before sonications, and 21–36 protein bands after repeated sonications. Therefore, for maximum efficacy, we mixed our mold antigens with commercially available allergenic mold extracts and used 2 µg/well of these mixtures on ELISA plates. With this mixture of mold antigens, we found that human sera diluted 1:100 for IgG, IgM, and IgA in serum diluent resulted in optimum ELISA optical densities. The coefficients of intra-assay and inter-assay variations were less than 16% for all isotypes. IgG antibodies to molds was the lowest (5.7–10.2%) and IgA the highest (5.6–11.3%).[100] Nonspecific proteins (HSA) did not change the levels of these antibodies; however, specific mold antigens extracted from *Stachybotrys* absorbed the IgG antibodies 43–58%, the IgM antibodies 21–38%, and the IgA antibodies 27–34%. Interestingly, when similar absorptions of sera were performed with other mold antigens, absorption of IgG, IgM, and IgA antibodies to *Stachybotrys* with *Alternaria* was insignificant, or less than 10%; however, reaction of the same sera with *Aspergillus* and *Penicillium* was capable of absorbing only the IgG antibody against *Stachybotrys* significantly (14–20%). This minor inhibition of IgG antibody against *Stachybotrys* with *Aspergillus niger* and *Penicillium notatum* antigens is an indication of antigenic cross-reactivity between these molds, which warrants further investigation. The significant inhibition of IgG, IgM, and IgA antibodies against *Stachybotrys* by specific molds and antigens (Table 6) provides further evidence for the specificity of these antibodies. Inhibition of these antibodies with mold antigens, along with the simultaneous presence of IgG, IgM, and IgA antibodies against different molds and satratoxin (Tables 1–4) leads us to conclude that these specific antibodies could be used in subpopulation studies and in epidemiologic investigations of mold and mycotoxin exposure. Furthermore, clinicians may want to consider possible mold exposure in patients who present with multiorgan system abnormalities.

Finally, the detection of IgG antibodies to satratoxin H reveals that the mycotoxin—or the spores and hyphae containing the mycotoxin—can behave as an antigen. This likely occurs by the combination of satratoxin H with carrier mold proteins, and their presentation to the cells involved in the immune system, resulting in subsequent antibody production. Similar observations have been reported for aflatoxin B1, patulin, and ochratoxin A used as a hapten.[107–112]

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