

Antibodies against Molds and Mycotoxins Following Exposure to Toxigenic Fungi in a Water-Damaged Building

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ABSTRACT. Exposure to molds in water-damaged buildings can cause allergy, asthma, hypersensitivity pneumonitis, mucus membrane irritation, and toxicity—alone or in combination. Despite this, significant emphasis has been placed only on Type I allergy and asthma, but not on the other 3 types of allergies. In this study, we sought to evaluate simultaneous measurements of immunoglobulin (Ig) G, IgM, IgA, and IgE antibodies against the most common molds, and their mycotoxins, cultured from water-damaged buildings. Antibodies against 7 different molds and 2 mycotoxins were determined by enzyme-linked immunosorbent assay (ELISA) in the blood of 40 controls and 40 mold-exposed patients. The IgG antibody levels against all 7 of the molds used, as well as the 2 mycotoxins, were significantly greater in patients than in controls. The IgM antibody levels were significantly different in patients for only 6 of 9 determinations. Regarding IgA determinations, antibodies were elevated significantly against all antigens tested, except *Epicoccum*. However, the differences in IgE levels in controls and mold-exposed patients were significant only for *Aspergillus* and satratoxin. These differences implied that, overall, the healthy control group was different from the mold-exposed patients for IgG, IgM, and IgA antibodies, but not for the IgE anti-mold antibody. Most patients with high levels of antibodies against various mold antigens also exhibited elevated antibodies against purified mycotoxins, indicating that the patients had been exposed to mold spores and mycotoxins. Detection of high levels (colony-forming units per cubic meter) of molds—which, in this study, strongly suggested that there existed a reservoir of spores in the building at the time of sampling—along with a significant elevation in IgG, IgM, or IgA antibodies against molds and mycotoxins, could be used in future epidemiologic investigations of fungal exposure. In addition to IgE, measurements of IgG, IgM, and IgA antibodies should be considered in mold-exposed individuals.

<Key words: antibodies, ELISA, mycotoxins, water-damaged buildings>

MOLDS represent a subgroup of fungi, which can grow where moist conditions exist (e.g., on wood, leather, carpets, clothing, drywall, sheet rock, and insulation). Given that molds grow in wet or moist indoor environments, individuals are sometimes exposed to molds or to their byproducts. Such exposures can occur either by direct contact with surfaces, or through the air if mold byproducts are aerosolized.¹ Exposure to certain

types of airborne molds and their spores can cause allergic reactions, episodes of asthma, and other respiratory problems in individuals who are predisposed genetically and immunologically.

Health problems that result from molds may also occur when individuals are exposed to large doses of chemicals called "mycotoxins," which are produced by molds.²⁻⁴ Mycotoxins are fungal metabolites that exert

toxic effects ranging from severe irritation to immunosuppression and cancer. Virtually all of the information related to disease induced by mycotoxins concerns ingestion of contaminated food.⁵ In heavily contaminated environments, neurotoxic symptoms related to airborne mycotoxin exposure have been noted.⁶

Several species of molds, including some found indoors in contaminated buildings, produce mycotoxins.⁷⁻⁹ During the past 10 yr, concern has escalated about exposure to multiple mycotoxins from a mixture of mold spores growing in wet indoor environments.¹⁰⁻¹² Adverse health effects from exposure to a mixture of mold spores are different from those related to a single mycotoxin exposure under controlled conditions. Indoor exposure to molds and their spores or mycotoxins more closely resembles the field exposure of animals to the entire mold or its byproducts than it does the controlled experimental laboratory exposure to a single agent via a single exposure route.^{13,14}

In contrast to a controlled laboratory exposure, field exposure of animals to molds produces effects on the immune system which are manifested as increased susceptibility to infectious diseases.¹³ Many mycotoxins are also cytotoxic; therefore, depending on the route of entry, mycotoxins may be damaging to the skin, lung, or gut. Such cytotoxicity to the lungs may (a) affect the physical defense mechanisms of the respiratory tract; (b) damage alveolar macrophages; and (c) decrease the airway's ability to clear bacteria, viruses, and other particulate matter in humans.¹³ The combined outcome of these immunosuppressive and cytotoxic activities of mycotoxins increases the susceptibility of the exposed person to infectious diseases, and possibly cancer.

Several toxigenic molds have been found by different laboratories during air-quality investigations of damp homes and offices. Among the genera found most frequently in numbers that exceed levels found outdoors are *Aspergillus*, *Penicillium*, *Stachybotrys*, *Chaetomium*, *Alternaria*, and *Cladosporium*.¹⁴⁻¹⁸ However, systematic studies of health effects and immunological reactions related to indoor exposure to these molds, and antibody production against their antigens and toxins, have not been studied simultaneously. Johanning et al.¹⁷ used *Stachybotrys* extracts and enzyme-linked immunosorbent assay (ELISA) and radioallergosorbent (RAST) protocols to measure mold-specific immunoglobulin (Ig)G and IgE in the blood of individuals exposed to toxigenic fungi. They found no elevated levels of these antibodies to *S. chartarum*, which was statistically significant.¹⁷ In another study, an increase in IgA production and IgA nephropathy was reported in mouse experiments after the rodents were injected with trichothecene or vomitoxin.¹⁹ Similar findings were reported when IgE, IgG, and IgA antibodies against *S. chartarum* were measured in patients with asthmatic or mycotoxicosis symptoms. 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 among the control groups. IgA and IgG levels were significantly higher ($p < 0.01$ and $p < 0.05$, respectively) in the patient group than in the control group. The IgE levels did not differ between groups. Raunio et al.²⁰ suggested that exposure to *Stachybotrys* did not cause IgE-mediated allergy in humans, and that the IgA response better reflected exposure to the fungus than did the IgG response.

Other investigators have reported that the serum IgA level was a more specific factor than IgG level for indicating Farmer's Lung disease, which is also associated with fungal exposure. Investigators reached the same conclusion concerning antibodies against *Aspergillus fumigatus*.^{21,22} When specific IgA was detected in a patient's sera, the IgA concentrations in bronchoalveolar lavage were high. These results suggest that the concentration of serum IgA may depend on the magnitude of respiratory exposure to fungi.²³

Given that the respiratory tract is the major route of exposure for fungi, their spores, and mycotoxins, we conducted the present study to measure specific IgG, IgM, IgE, and IgA in blood against 7 different molds found most frequently in water-damaged buildings. Moreover, we analyzed these antibodies against molds, as well as against 2 of the most common mycotoxins they produce, to develop a more useful biomarker of exposure in the work environment. Simultaneous detection of antibodies against both toxigenic fungi and their mycotoxins may indicate occupational exposure to molds and their metabolites in a subpopulation of individuals exposed to molds.

Method and Materials

Environmental exposure evaluation and patients. The study population of 40 patients (e.g., executives, directors, secretaries, and administrators [among others], who ranged in age from 26 yr to 62 yr of age [11 males and 29 females]) worked for various organizations and was exposed occupationally to molds under the same indoor environmental conditions. Most subjects had moved to offices in the building in 1995 or earlier. In December 1998, major flooding occurred, and water entered the roof of the building. The flood scenario was described as "waterfalls from the ceiling"; the building and its fittings became damp, moist, and broken. Carpets felt "spongy" when walked upon. Attempts were made to patch the roof; however, it continued to leak during the year 2000. Commencing in the summer of 2001, patients could see colorful molds growing on the walls, and portions of the ceiling began to peel. The musty odor that pervaded the building was so unpleasant that the employees chose to wear paper masks. Soon after these events occurred, employees began to seek medical help.

Each employee was administered a health survey—during which a questionnaire developed by the American College of Occupational and Environmental Medicine (Arlington Heights, Illinois) was administered and a complete physical examination was conducted. Neurological and behavioral symptoms—including blurred vision, memory loss, fatigue, headache, migraine headache, nausea, loss of balance, cognitive deficits, rhinitis, sinusitis, nosebleeds, rashes, allergies, and painful lymph nodes—were recorded. Reported symptoms were similar to those indicated by patients with Chronic Fatigue and Fibromyalgia syndromes. Some of these symptoms were confirmed by pulmonary function testing and single-photon emission computed tomography (SPECT) scan. Environmental engineering firms tested the building (i.e., tape transfer, swab samples, and viable microbial activities were performed at different locations, including walls, ceilings, tiles, and sinks), and elevated levels (i.e., > 2,000 CFU/swab) of *Aspergillus*, *Alternaria*, *Cladosporium*, *Penicillium*, *Stachybotrys*, and *Chaetomium* were reported. Blood samples were drawn from the patients and from 40 age- and sex-matched control subjects who had no history of exposure to toxigenic indoor molds or symptoms associated with such exposure. The samples were sent to our laboratory by overnight courier, maintained at 4 °C, and subjected to various examinations (described later).

All patients gave their informed consent and allowed inclusion of their data in this manuscript without disclosure of their identity in the publication. Furthermore, we removed the specimens' identifier numbers from the samples, labeled them 1–40, and maintained them at –20 °C. A few months later, levels of IgG, IgM, IgA, and IgE antibodies against 9 different mold antigens were measured. Inasmuch as residual serum material was examined retrospectively, and at the time of examination samples did not have patient/donor identifiers, this study was exempt from oversight by the institutional review board.

Preparation of fungal antigen. The protocol for optimal fungal antigen extract preparation was followed, using procedures described previously.^{24–27} Molds, *Stachybotrys chartarum*, *Chaetomium globosum*, *Penicillium notatum*, *Aspergillus niger*, *Alternaria alternata*, *Cladosporium herbarum*, and *Epicoecum nigrum* were obtained from the American Type Culture Collection (Rockville, Maryland). Initially, the molds were cultured on 2% malt extract agar (MEA) for 8 days at 25 °C, after which spore suspensions were prepared in 0.1 M phosphate buffered saline (PBS) (pH 7.4) that contained 0.05% Tween 20™ (i.e., sorbitan mono-9-octadecenoate poly[oxy-1,1-ethanediyl]). For each spore suspension, 1 ml was inoculated into 100 ml of 2% malt extract broth (for *Stachybotrys*), or into cellulose broth (for the others) in untreated glass bottles, and the cultures were incubated for 10 days at 25 °C. 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 were suspended at 50 mg/ml in 0.1 M PBS (pH 7.5) containing 0.02% phenylmethyl sulfonyl fluoride (PSF) and 0.02% sodium azide. The mycelium suspension was sonicated for 5 sec at an output of 70% with Virsonic 50 (Virtis Company [Gardiner, New York]). The sonication step was repeated 10 times for maximum cell disruption; thereafter the suspension remained on a shaker for 24 hr at 4 °C. Following 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 its components were analyzed by 15% sodium dodecyl sulfate (SDS) gel electrophoresis (Bio-Rad Laboratories [Hercules, California]).

Preparation of mycotoxins. Mycotoxins from different molds were prepared in accordance with a modified method that originated in our laboratory.¹⁷ A total of 100 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 (Bio-Rad Laboratories [Hercules, California]) 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 (HPLC) (ESA [Chelmsford, Massachusetts], model 5600 Coularray Detector, with solvent delivery pump model 580 and an analytical cell that uses 2 porous graphite electrodes). The column was C-18 rainin 5- μ m 4.6 $\times$ 250 mm, with a 15-min gradient of 60–75% methanol in water, a flow rate of 1 ml/min, and monitoring at 260 nm. Two peaks—1 at 10.6 min and the other at 12.2 min—were obtained which corresponded in retention time to those of satratoxin H and trichothecene. The total amounts of satratoxin H and trichothecene were obtained in 100 mg of sample and were estimated at approximately 1.5 μ g and 1.7 μ g, respectively.

Binding of mycotoxins to human serum albumin (HSA). Satratoxin was coupled to the carrier protein HSA with 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide-metho-4-toluolsulfonate (CCMT) and with succinic anhydride. A total of 100 μ g of satratoxin in 100 μ l pyridine was reacted with 2 mg of succinic anhydride in a 45 °C water bath with off-and-on vortexing. The reaction mixture was evaporated to dryness. The satratoxin-hemisuccinate was dissolved in 200 μ l of dimethyl formamide and was added dropwise to a 1-ml solution of 0.1 M carbonate buffer (pH 9.5) that contained 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 with 1 M HCl, followed by an additional incubation of 4 hr. Finally, after the addition of 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 g against 0.1 M PBS (pH 7.2) for 48 hr. Following 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, IgA, and IgE against fungal antigens and mycotoxins. We analyzed levels of IgG, IgM, IgA, and IgE against antigens of molds in human sera by indirect ELISA. Microtiter plates were coated with 0.1 ml of HSA in duplicate, to serve as controls, or with mold extract and mycotoxins at a protein concentration of 10 µg/ml. Following incubation, washing, and blocking with 2% bovine serum albumin (BSA), 0.1 ml of human serum, at an 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 duplicate wells of the plates. Plates were incubated at 37 °C for 2 hr and washed 3 times with PBS Tween 20 (PBST), after which 0.1 ml of affinity-purified goat anti-human IgG(γ), IgM(μ), IgA(α), or IgE(ε) light-chain-specific, conjugated with peroxidase at dilutions of 1:500, were added and incubated at 37 °C for 1 hr. Following 4 washings with PBST and the addition of 0.1 ml of 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate, color development was measured. After incubation for 30 min, and addition of a stop solution, the intensity of color was measured spectrophotometrically at 492 nm. For each specimen, the ELISA background reading of wells coated with HSA was automatically subtracted from the wells coated with mold antigens. The background reading of wells coated with nonspecific antigen (i.e., HSA) reacted with the serum and all other reagents and was less than 12% of the absorbances of the wells coated with mold antigens. In each assay, serially diluted sera from rabbits immunized with molds antigens, and from patients after immunotherapy with high levels of antibodies against different molds, were used for the construction of a standard curve. We plotted the mean absorbances obtained from duplicate wells from each calibrator against their optical density (OD) values to determine the ODs for unknown samples.

Coefficients of intra-assay and inter-assay variations. We calculated coefficients of intraassay variations by running 5 samples 8 times in 1 assay. Coefficients of interassay variations were determined by measuring the same samples in 6 consecutive assays. This replicate testing (a) established the validity of the ELISA assays;

(b) determined the appropriate dilution with minimal background; and (c) detected serum, IgG, IgM, IgA, and IgE against different antigens. Two sera from healthy controls and 2 from patients exposed to molds were used for the construction of control curves.

Determination of optimal dilution of serum. We determined the optimal dilutions of sera by diluting 20 different controls' and patients' sera 1:25–1:400 in serum diluent buffer and adding the sera 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.

For IgE assay, 20 different sera from controls and patients were used undiluted or diluted from 1:2 to 1:8 in serum diluent buffer. All other steps were similar to IgG, IgM, or IgA determinations. Good linearity was maintained throughout the IgE calibration range. However, given that a dilution of 1:2 provided minimal background, it was used in the IgE assay.

Antibody specificity testing by absorption of sera. Specificity of the ELISA assay for molds and mycotoxin antibodies was confirmed by mold antigens or mycotoxin competition. For this testing, 6 different sera with high levels of IgG, IgM, and IgA antibodies (OD in ELISA > 0.8) against *Stachybotrys* or satratoxin were used in different test tubes. One ml of each serum was preincubated with 1 mg of HSA, *Stachybotrys* antigens, satratoxin and trichothecene bound to HSA, or 10 ng of each mycotoxin alone. After mixing, 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 and mycotoxins.

Statistical analysis. Systat program version 5.2 (Systat, Inc. [Evanston, Illinois]) was used for statistical analysis. Normal distribution of data was tested by the Kolmogorov-Smirnov 1-sample test. One-way analysis of variance (ANOVA) was performed with ANOVA and the Kruskal-Wallis H test for ranked data. For post hoc analysis, Tukey's test was employed. Correlations were performed with Pearson's test and the nonparametric Spearman's rank order test. In this study, *p* values ≤ 0.05 were considered significant.

Results

Fungal contamination in a water-damaged building. Environmental laboratory examinations of the water-damaged office building were conducted by an environmental engineering firm. Microscopic and culture analysis revealed significant fungal contamination with *Alternaria*, *Aspergillus*, *Chaetomium*, *Cladosporium*, *Epicoccum*, *Penicillium*, and *Stachybotrys*. Concentration samples of these molds were taken from 7 different

locations in the building. In 4 of 7 swab samples, viable microbial activity on MEA and cellulose agar exceeded 2,000 colony-forming units (CFU/swab) (Table 1). Sample 2 was dominated by *Stachybotrys*; sample 4 by *Penicillium* and *Chaetomium*; sample 5 by *Epicoccum* and *Penicillium*; and sample 6 by *Penicillium* and *Epicoccum*. Total bioaerosols were also measured by collecting air samples and culturing on MEA and cellulose agar. Results of 4 different samples showed a range of 42–32,251 CFU/m³ of molds, with *Cladosporium* being the predominant species (Table 2). In air samples 1 and 3, the concentration of *Cladosporium* was approximately 1×10^3 and 3×10^4 CFU/m³, respectively (Table 2). Detection of a significantly high level (> 400 CFU/m³) of molds strongly suggested that reservoirs of spores existed in the building at the time of sampling. Patients reported to their examining physicians the presence of a “strong musty odor.” In fact, they stated that the musty odor was so offensive that they began wearing masks, which allowed them to function more effectively. Soon afterwards, they presented with neurological and behavioral symptoms. The most common symptoms were fatigue, headache, easily losing one’s breath, muscle and joint pain, memory disturbance, multiple chemical sensitivities, and allergies. Some of these abnormalities were confirmed by pulmonary function tests and SPECT scans, which were abnormal in 60% and 80% of patients, respectively (Table 3). These symptoms were present in a statistically significant ($p < 0.0001$) larger proportion (55–92.5%) of em-

ployees in the contaminated office building than in controls (0–25%).

Detection of IgG, IgM, IgA, and IgE antibodies. Sera from healthy control subjects who had no history of exposure to molds, and from 40 patients exposed to molds or their spores in the indoor environment, were analyzed for the presence of IgG, IgM, IgA, and IgE antibodies against different mold antigens and mycotoxins. The ELISA results were expressed by individual and mean $\pm$ standard deviation (SD) of OD at 492 nm. The OD for IgG antibody values obtained with a 1:100 dilution of healthy control sera ranged from 0.01 to 1.27 OD and varied among subjects and mold antigens. The mean $\pm$ SD of these OD values ranged from 0.16 ± 0.14 OD to 0.24 ± 0.26 OD. The corresponding IgG OD values from mold-exposed patients’ sera ranged from 0.02 to 1.97 OD; mean $\pm$ SD levels for IgG values varied from mold to mold and ranged from 0.30 ± 0.25 OD to 0.52 ± 0.53 OD. For all 7 molds and the 2 mycotoxins, the difference between mean $\pm$ SDs of controls’ sera and mold-exposed patients’ sera were statically significant (*Alternaria*: $p = 0.008$; *Aspergillus*, *Stachybotrys*, and *Chaetomium*: $p = 0.001$; *Cladosporium*: $p = 0.009$; *Epicoccum*: $p = 0.003$; *Penicillium*: $p = 0.0005$; satratoxin: $p = 0.013$; and trichothecene: $p = 0.049$ [Figs. 1–4]).

At OD values above the mean of 2 standard deviations in the healthy control group, levels of IgG antibodies against these antigens were calculated in controls’ and patients’ sera, and we found that even though 10–15% of the controls’ sera had IgG values that were 2

Table 1.—Tape Transfers and Microscopic Examination of Bulk Samples and Swab Samples for Viable Microbial Activity on Malt Extract Agar and Cellulose Agar

Sample no.	Results of tape transfer and microscopic mold identification	Percentage	Swab samples for viable microbial activity	
			Mold identification	CFU/swab
1	<i>Stachybotrys</i> spores Amorphous dirt and dust	96 4	Viable molds	0
2	<i>Stachybotrys</i> spores Amorphous dirt and dust	98 2	<i>Stachybotrys</i> sp.	$> 2,000$
3	<i>Chaetomium</i> spores and hyphae Cellulose fiber Amorphous dirt and dust	60 20 20	Viable molds	0
4	<i>Chaetomium</i> spores <i>Penicillium</i> and <i>Aspergillus</i> spores Amorphous dirt and dust	80 15 5	<i>Chaetomium</i> sp. <i>Penicillium</i> sp.	$> 2,000$ $> 2,000$
5	<i>Alternaria</i> spores Amorphous dirt and dust	95 5	<i>Epicoccum</i> sp. <i>Penicillium</i> sp.	$> 2,000$ 270
6	<i>Chaetomium</i> spores and hyphae Amorphous dirt and dust	20 80	<i>Epicoccum</i> sp. <i>Penicillium</i> sp.	560 $> 2,000$
7	<i>Stachybotrys</i> spores Amorphous dirt and dust	0 100	Viable molds	0

Note: CFU = colony-forming units.

standard deviations higher than the mean control group, the mold- and mycotoxin-exposed group revealed an elevation in IgG values from 20% to 42.5% ($p < 0.01$ [Table 4]). The individual data presented in Figures 1–4 showed that if specimens from controls and patients presented an elevation in IgG, IgM, IgA, and IgE antibodies against 1 or more molds, they also showed a significant elevation in antibodies against mycotoxins. However, we found several specimens that were positive for IgG antibodies against 1 or more molds that did not have any

Table 2.—Air Samples for Viable Microbial Activity on Total Bioaerosols

Sample no.	Mold identification	CFU/m ³
1	<i>Cladosporium</i>	1,140
	<i>Aspergillus</i>	340
	<i>Penicillium</i>	340
	Myxomycetes, smuts	160
	Others	<100
	Total count	2,200
2	<i>Cladosporium</i>	390
	Ascospores	30
	Hyphae fragments	60
	Myxomycetes, smuts	30
	Total count	510
3	<i>Cladosporium</i>	31,550
	Myxomycetes, smuts	414
	<i>Leptosphaeria</i>	234
	<i>Alternaria</i>	18
	Bipolaris	17
	Ascospores	18
	Total count	32,251
4	<i>Cladosporium</i>	28
	Myxomycetes, smuts	14
	Total count	42

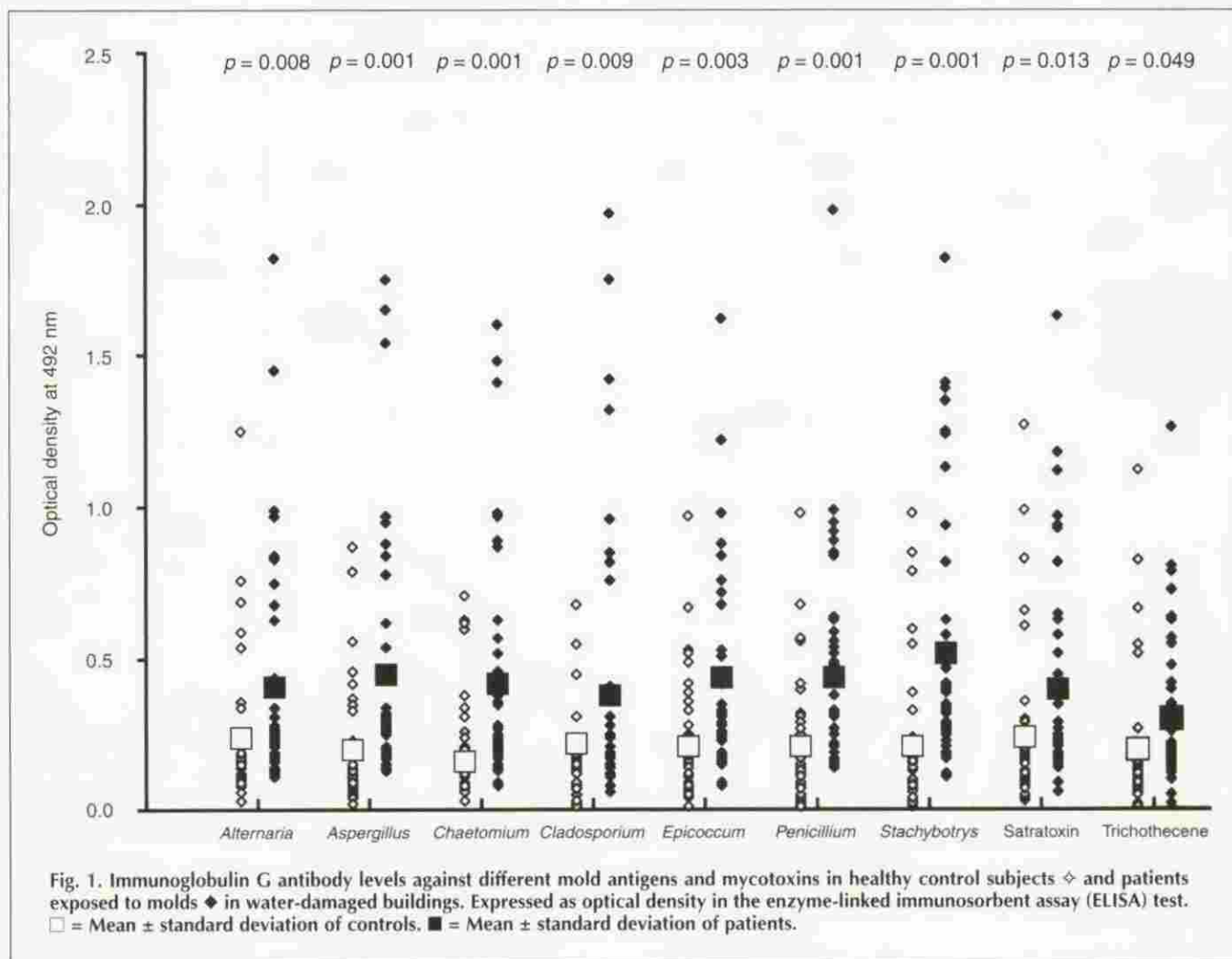
antibodies against satratoxin or trichothecene. Levels of IgA antibodies against molds and mycotoxins in sera of healthy controls and mold-exposed patients are shown in Figure 2. These serum IgA antibodies against all 7 molds and the 2 mycotoxins were significantly higher in patients than in controls. The mean $\pm$ standard deviation for controls ranged from 0.17 ± 0.16 OD to 0.27 ± 0.29 OD; for patients, these values ranged from 0.35 ± 0.34 OD to 0.67 ± 0.70 OD. Differences between IgA antibody levels in controls and patients were statically significant (i.e., *Alternaria*: $p = 0.008$, *Aspergillus*: $p = 0.01$, *Chaetomium*: $p = 0.006$, *Cladosporium*: $p = 0.007$, *Penicillium*: $p = 0.015$, *Stachybotrys*: $p = 0.002$, satratoxin: $p = 0.018$, and trichothecene: $p = 0.004$). With respect to *Epicoccum*, however, the differences in IgA levels were not significant ($p = 0.25$). When we used 2 standard deviations of the mean control as the cutoff point, 10–17.5% of controls vs. 22.5–42.5% of mold-exposed patients showed elevated IgA antibody levels ($p < 0.01$). The highest percentage of IgA antibody levels was initially attributed to *Stachybotrys*—followed by *Penicillium* and *Chaetomium* (Table 3).

Likewise, IgM antibody levels against these molds and mycotoxins were examined in both groups. The mean $\pm$ standard deviation of the data showed significant differences between control and patient groups only for *Stachybotrys*, *Cladosporium*, *Alternaria*, *Aspergillus*, satratoxin, and trichothecene ($p < 0.05$)—but not for *Chaetomium*, *Epicoccum*, or *Penicillium*. The percentage of elevated serum IgM antibodies against these molds and mycotoxins were elevated more significantly in patients than in controls for *Stachybotrys*, satratoxin, *Cladosporium*, and *Penicillium* (Table 3). Similar to IgG anti-mold antibody values, IgA and IgM

Table 3.—Percentage of Different Symptoms or Abnormal Examinations in Controls ($n = 40$) and Patients Exposed to Molds ($n = 40$)

Symptom	Percentage positive in controls	Percentage positive in mold-exposed patients
Fatigue	20.0	92.5
Memory disturbance	10.0	72.5
Depression	10.0	52.5
Anxiety	12.5	52.5
Headaches	22.5	87.5
Blurred vision	0.0	62.5
Lightheadedness	15.0	60.0
Ringing in ears	5.0	62.5
Muscle and joint aches	25.0	75.0
Recurrent flu-like illnesses	5.0	55.0
Severe allergies	17.5	67.5
Chest pain and cough	10.0	70.0
Out of breath easily	15.0	80.0
Multiple Chemical Sensitivity	25.0	72.5
Pulmonary function test	0.0	60.0
SPECT scan	0.0	85.0

Note: SPECT = single-photon emission computed tomography.



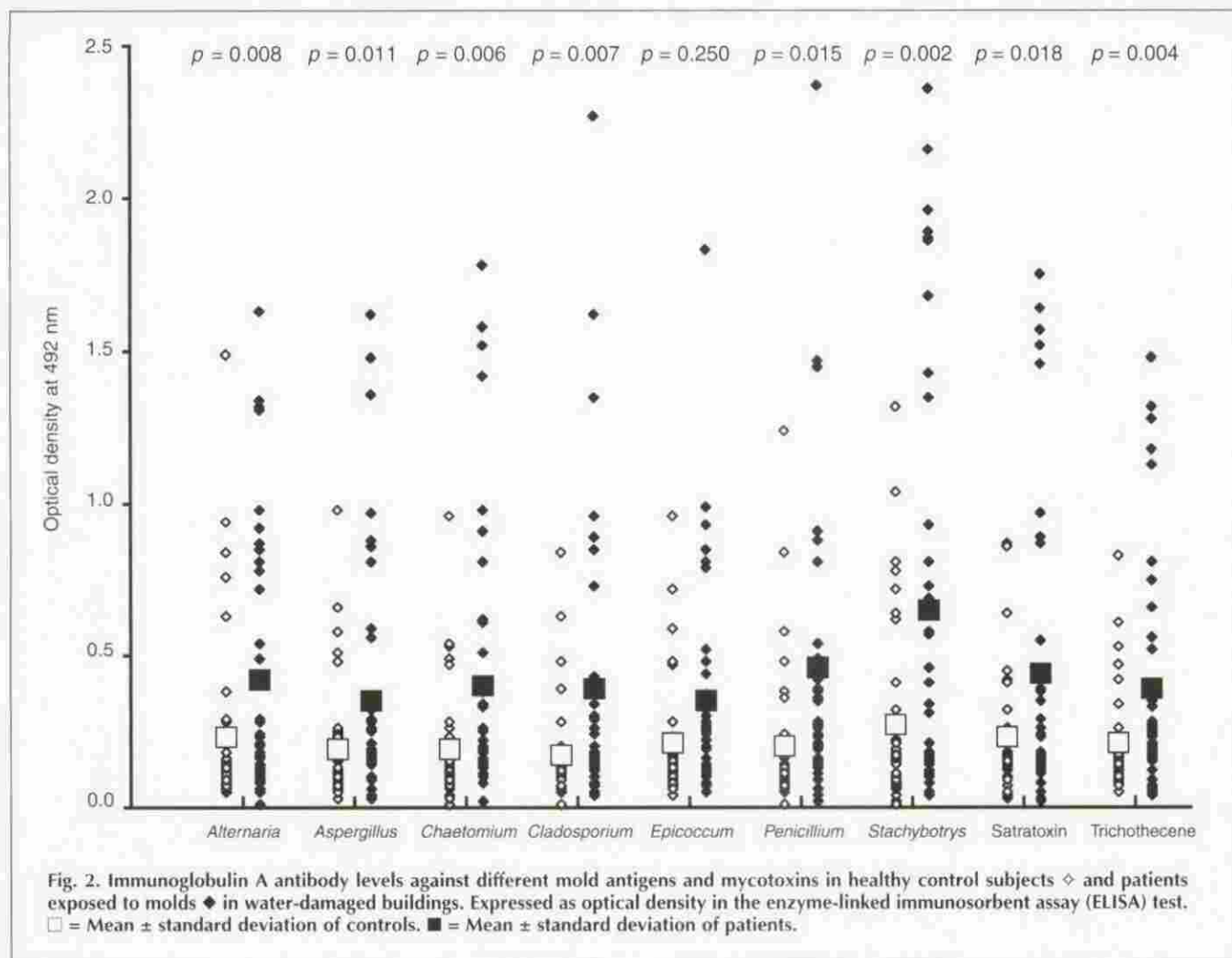
levels against mycotoxins were elevated only when the same specimens had high levels of antibodies against 1 or more molds.

Fungal-specific IgE antibodies were measured using similar microplates coated with fungal antigens and mycotoxins. Each serum was assayed at a 1:2 dilution in serum diluent, in duplicate. The mean $\pm$ SD for IgE levels is shown in Figure 4. Whereas the overall mean $\pm$ SD for IgE values was higher in patients than in controls, only *Aspergillus* and satratoxin values reached a statistically significant level of $p \leq 0.05$; for other molds and mycotoxins, p values exceeded 0.05. However, at cutoff values of 2 standard deviations, 7.5–12.5% of control sera and 12.5–27.5% of mold-exposed sera of patients revealed elevated IgE values (Table 4).

Specificity, intra-assay, and inter-assay precision. The coefficient of intra-assay variation calculated for 5 samples from the results of 8 replicates was 5.7–10.2% for IgG, 5.8–8.2% for IgM, 5.6–11.3% for IgA, and 7.7–10.9% for IgE. The 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%,

10.6–15.3%, and 11.1–18.5%, respectively, for IgG, IgM, IgA, and IgE. For testing of specificity of IgE antibodies, 10 different sera, with ODs between 0.32 and 1.98, were examined simultaneously by commercially available mold-specific IgE ELISA assay. Compared with the ELISA described in the Method and Materials section, the overall correlation was 88%.

Absorption of *Stachybotrys* antibodies with HSA. *Stachybotrys* mycotoxins bound to HSA were used in inhibition studies and for examination of antibody specificity. Three different sera with high levels of IgG, IgM, or IgA against *Stachybotrys*, and 3 sera with high levels of antibodies against satratoxin and trichothecene were absorbed with nonspecific and specific antigens. Results showed that nonspecific proteins—such as HSA—did not change the IgG, IgM, and IgA antibody levels against *Stachybotrys* and mycotoxins; however, *Stachybotrys* antigens, satratoxin, satratoxin-bound HSA, trichothecene, or trichothecene-bound HSA absorbed the IgG antibody levels from 42–61%, 15–40%, 33–44%, 12–34%, and 24–51%, respectively. Similar to IgG—but to a lesser degree—IgM and IgA antibodies were absorbed more effectively with *Stachybotrys* antigens than



with mycotoxins bound to HSA or with mycotoxins alone. This significant absorption and inhibition of IgG, IgM, and IgA antibodies by fungal antigens or mycotoxins bound to carrier proteins is the best evidence for the specificity of fungal antibodies.

Discussion

Health effects from exposure to molds in water-damaged building environments can result in allergy, infections, or mucous membrane and sensory irritation toxicity alone, or in combination. However, despite this variety of adverse health effects, significant emphasis is placed only on Type I allergy.²⁹⁻³¹

There are hundreds of molds with thousands of different antigens that can contaminate indoor air. Depending on the types of molds and antigens, as well as an individual's genetic susceptibility, 1 or all 4 types of allergic reactions are possible following exposure. This means that atopic individuals exposed to molds found indoors can develop sensitization and allergy, which can be confirmed by a skin test or RAST IgE test. However, the nonatopic individual, as a result of a lack of

IgE production, would not be identified as having mold allergy, even while presenting symptoms of chronic illnesses.³² For this reason, mechanisms other than IgE mediation have been explored by various investigators. In an epidemiological and immunological study of materials from a water-damaged office building, samples were contaminated with *Stachybotrys atra* containing mycotoxins. Employees exhibited low levels of abnormalities—in both their cellular and humoral immunities. Although 48 individuals were possibly exposed to *Stachybotrys*, only 4 had an elevated antibody test for it.¹⁷ Other investigators in several case-control studies have used serology in investigations of subjects from buildings with large amounts of *S. chartarum* and have found statistically nonsignificant differences in *S. chartarum* IgG or IgE antibody levels between exposed and control groups.¹⁶⁻¹⁸ Conversely, a recent cross-sectional comparison study of water-damaged or mold-contaminated homes in Finland found fungal-specific IgG concentrations in the sera of residents in houses with and without mold, with other cases showing a tendency for higher antibody levels to most fungi than in control groups.³³

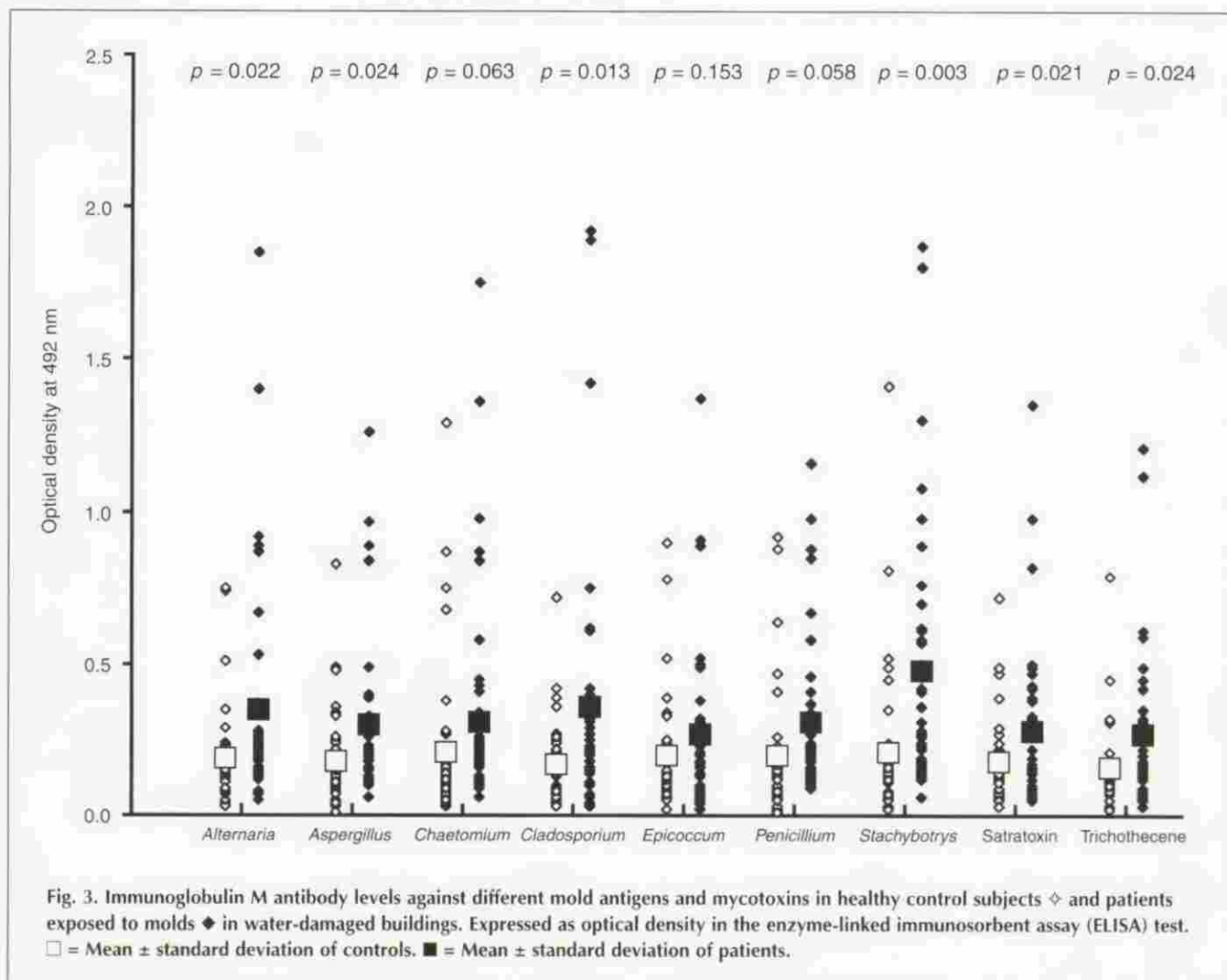


Fig. 3. Immunoglobulin M antibody levels against different mold antigens and mycotoxins in healthy control subjects $\diamond$ and patients exposed to molds $\blacklozenge$ in water-damaged buildings. Expressed as optical density in the enzyme-linked immunosorbent assay (ELISA) test. $\square$ = Mean $\pm$ standard deviation of controls. $\blacksquare$ = Mean $\pm$ standard deviation of patients.

In another study, following administration of both strains of *S. atra* and spores, minor changes in hematological parameters were detected in mice ($p < 0.05$); however, IgG antibodies to *S. atra* could not be detected in the sera of mice exposed to *S. atra* spores intranasally.³⁴

Raunio et al.²⁰ measured IgE, IgG, and IgA antibodies against *S. chartarum* in patients with asthmatic or mycotoxicosis symptoms. They reported that there was a better correlation between the IgA response and exposure to the fungus than was found with the IgG response. For the reasons stated earlier, we tested IgG, IgA, IgM, and IgE antibodies against the 7 most common molds found in water-damaged buildings, as well as against 2 different mycotoxins produced by some of these molds, including *Stachybotrys*.

Data presented in Table 4 showed that at a cutoff point of 2 standard deviations above the mean of controls, a significant percentage of controls had elevated IgG, IgM, and IgA antibodies against mold and mycotoxins. This elevation of mold-specific antibodies in healthy controls is an indication of widespread expo-

sure of the population to indoor molds. At the time of venous blood drawing, these individuals did not present the neurological and behavioral symptoms found in patients exposed to toxigenic molds. Furthermore, presentation of clinical symptomatology did not correlate with the levels of mold antibodies in all patients. Therefore, it seems that antibody levels do not correlate with disease severity, but are indicative of exposure. Therefore, in some individuals, detected mold IgG, IgM, and IgA antibodies may be protective—but not pathogenic. The question remains: "Why are antibody levels not correlated with disease?" Genetics and epidemiological studies suggest that "You and I can be exposed to exactly the same amount of antigens or haptenic chemicals, but our responses will differ, perhaps by a hundred-fold."³⁵⁻³⁷ This variation in the ability to handle antigens and haptenic chemicals depends on genetic susceptibility and enzyme polymorphism. In this regard, numerous specific genetic polymorphisms in multigene families of cytochrome P450 and glutathione s-transferase have been described in humans during the past decades.³⁶

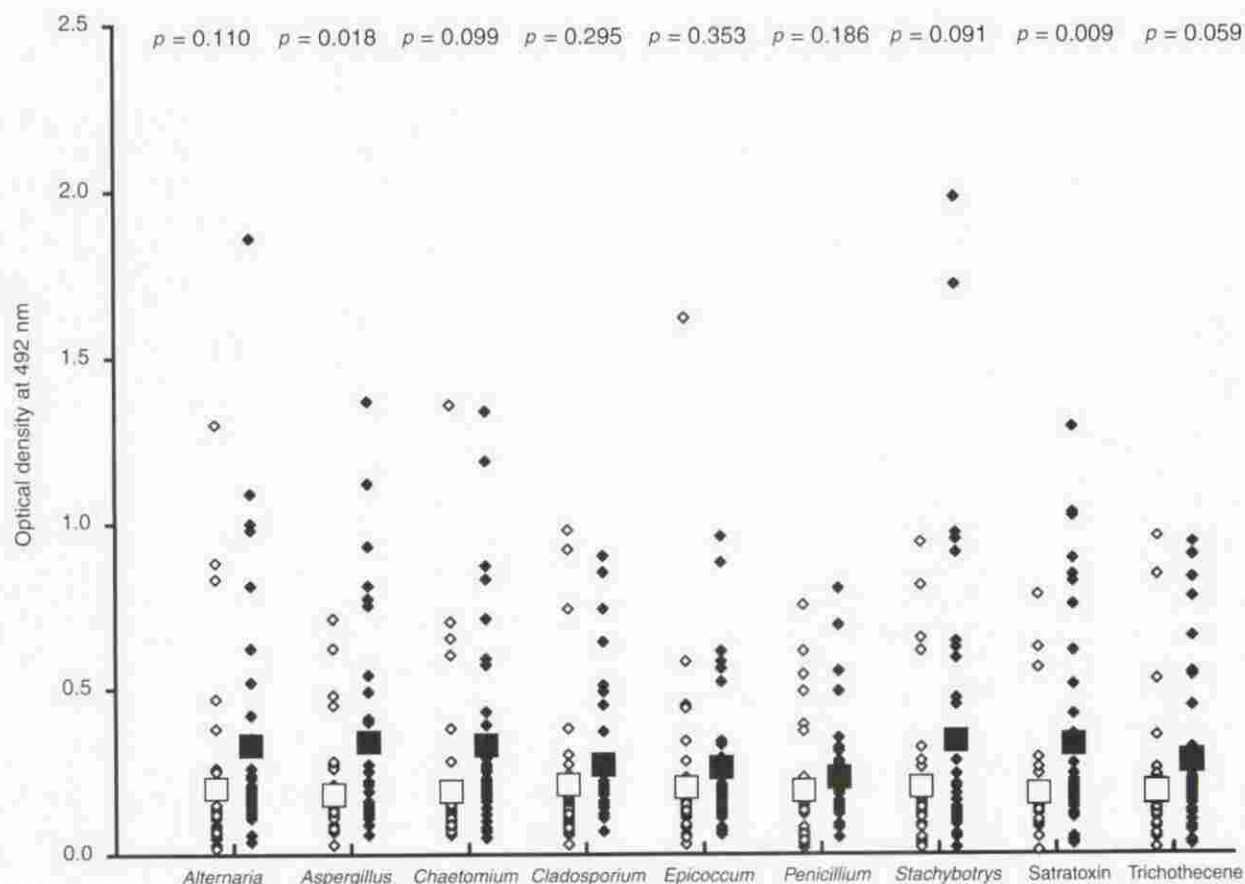


Fig. 4. Immunoglobulin E antibody levels against different mold antigens and mycotoxins in healthy control subjects $\diamond$ and patients exposed to molds $\blacklozenge$ in water-damaged buildings. Expressed as optical density in the enzyme-linked immunosorbent assay (ELISA) test. $\square$ = Mean $\pm$ standard deviation of controls. $\blacksquare$ = Mean $\pm$ standard deviation of patients.

Table 4.—Percentage Elevation of Antibodies against Different Molds and Mycotoxins above the Mean (± 2 Standard Deviations) of Control Optical Density in Healthy Controls and Patients Exposed to Molds

Molds and mycotoxins	Immunoglobulin							
	G		A		M		E	
	Control	Patient	Control	Patient	Control	Patient	Control	Patient
<i>Alternaria</i>	15.0	25.0	12.5	35.0	7.5	15.0	10.0	20.0
<i>Aspergillus</i>	12.5	30.0	12.5	22.5	7.5	20.0	10.0	25.0
<i>Chaetomium</i>	15.0	42.5	12.5	30.0	10.0	22.5	10.0	25.0
<i>Cladosporium</i>	10.0	20.0	10.0	30.0	10.0	25.0	7.5	15.0
<i>Epicoccum</i>	12.5	30.0	12.5	22.5	7.5	15.0	10.0	15.0
<i>Penicillium</i>	10.0	40.0	10.0	30.0	12.5	20.0	12.5	12.5
<i>Stachybotrys</i>	12.5	35.0	17.5	42.5	12.5	40.0	10.0	27.5
Satratoxin	12.5	27.5	7.5	22.5	10.0	27.5	7.5	25.0
Trichothecene	12.5	25.0	10.0	25.0	5.0	20.0	7.5	20.0

In the health-effect survey, investigators reported that in 56% of workers exposed to toxigenic fungi, 33% of the controls had Multiple Chemical Hypersensitivity.¹⁷ In our questionnaire, 72.5% of mold-exposed vs. 25% of controls reported chemical sensitivity (Table 2). Therefore, a high percentage of controls, who had high

levels of antibodies against molds and mycotoxins, may not be genetically susceptible to mold antigens and mycotoxins and, hence, do not show the same health-effect responses as do a selective number of workers exposed to toxigenic fungi. In addition, we have no information about the degree or level of exposure to

fungal antigens in the control group, or whether individuals with high levels of antibodies will develop symptomatology in the future. Moreover, the fungal antibodies found in healthy controls could be protective or cross-reactive, inasmuch as some fungal antigens may cross-react with one another,³⁸ and sometimes they even completely cross-react with different antigens (e.g., *Aspergillus* and human myelin basic protein³⁹ or *Aspergillus* and superoxide dismutase homology⁴⁰). Despite these cross-reactive or nonspecific antibodies in healthy controls, data presented in Figures 1–4 reveal significant differences in the mean $\pm$ standard deviation of controls vs. patients.

Overall, for the majority of tested molds and mycotoxins, the IgA and IgG levels were significantly higher in the patients than in the control group ($p < 0.01$). In patients, the IgM antibody levels against *Stachybotrys*, *Cladosporium*, *Alternaria*, *Aspergillus*, and mycotoxins—but not against *Chaetomium*, *Epicoccum*, and *Penicillium*—were significantly different from levels in controls ($p \leq 0.05$). With respect to IgE values, whereas the overall mean $\pm$ SD was higher in patients than controls, only antibodies to *Aspergillus* and satratoxin reached a statistical significance level of $p \leq 0.05$ (Fig. 4). Similar to the results of earlier studies,^{16–18} we found nonsignificant differences in *Stachybotrys* IgE antibody levels in exposed and control groups ($p = 0.09$). In our study, the increase in IgE antibody levels against *Aspergillus* and satratoxin may be explained on the basis of recent studies in mice^{41–43} and humans,^{44,45} both of which have shown that a TH₁/TH₂ dysregulation and switch to TH₂ immune response is responsible for hyperimmunoglobulin E syndrome.⁴⁶

Whereas our overall antibody test results against mold antigens correlated with the IgG and IgA antibody levels against *Stachybotrys* reported earlier,²⁰ differences in antibody levels may stem from antigen preparation and dilution of serum.^{17,18} Other investigators cultured the fungus and suspended mycelium in PBS containing PSF, extracted the antigens by homogenization and incubation on the shaker for 16–18 hr at 4 °C, and used human sera at dilutions of 1:10, 1:40, or 1:25, for IgE, IgG, and IgA analysis, respectively.²⁰ One weakness of the aforementioned studies—ours included—stems from a lack of examination of antibodies against molds not isolated from the building(s) and grown under the same conditions.

We prepared our fungal antigens similar to the method described above, but with some modification.²⁰ We used similar culture and suspension techniques, but we used repeated sonications during 24 hr of extractions. This resulted in a higher yield of fungal antigens in suspensions. We then compared the protein concentration of commercially prepared fungal antigens with our antigen preparations before and after sonication. The protein concentration of commercial antigens

ranged from 0.4 to 2.1 mg/ml, whereas in our preparations, protein concentrations ranged from 0.8 to 3.5 mg/ml prior to sonication. After sonication for 10 times, the concentration of proteins increased to 5.8–16.5 mg/ml. Moreover, by performing SDS gel electrophoresis on an equal amount of protein (i.e., 1 mg [depending on mold species]), we detected between 9 and 22 bands in commercial antigens, between 7 and 16 bands in our preparations before sonications, and between 21 and 36 protein bands after repeated sonications (data not shown). Therefore, we used these antigens with a maximum number of fungal antigens at a concentration of 1 μ g/well on ELISA plates and found that human sera—dilutions of 1:2 for IgE and 1:100 for IgG, IgM, or IgA in serum diluent—resulted in optimal ELISA optical densities.

The determination of IgG, IgM, and IgA antibody levels, at sera dilutions of 1:100, was based on hundreds of ELISA assays performed in our laboratory. Also, many commercially available ELISA kits use dilutions of either 1:100 or 1:200 for the determination of IgG antibodies in serum. Moreover, in this study, 20 different sera diluted at 1:25, 1:50, 1:100, 1:200, and 1:400 were measured for antibody levels. For all tested sera, the curve at dilutions of 1:50 to 1:200 was linear and, therefore, the IgG, IgM, and IgA antibody detection was performed at 1:100 dilution of serum.

Given the isotype competition from IgG, IgM, and IgA with serum concentrations of 0.5–16 mg/ml, the ideal method for measuring the IgE level is to capture all IgE from a sample in which anti-IgE antibodies are used, or to remove the other immunoglobulins from the serum; for example, absorption of the serum by anti-IgG, IgM, IgA, or with Protein A.⁴⁷ However, given that the antibodies used in our ELISA assays are specific and affinity-purified, exhibiting no cross-reactivity to other immunoglobulin classes, we did not perform any absorption studies for IgE assay.

This simultaneous detection of 1 or more classes of antibodies against all molds and mycotoxins suggests cross-reaction between different mold antigens, as has been shown in earlier studies.³⁸ In addition, simultaneous detection of antibody classes against 2 different mycotoxins (satratoxin and trichothecene) indicates that spores containing mycotoxins can behave as an antigen and, by their presentation to the cells involved in the immune system, result in antibody production against mold antigens—as well as metabolites such as mycotoxins.

To our knowledge, there has been no study concerning the effects of fungal exposure on serum IgG, IgA, IgM, and IgE levels against several molds' antigens, in particular against the mycotoxins. These antibodies appear to be specific, inasmuch as in our absorption studies only specific antigens such as *Stachybotrys*, or mycotoxins bound to HSA, could reduce the antibody

levels against *Stachybotrys*, satratoxin, or trichothecene by up to 60%, whereas free satratoxin and trichothecene were significantly less effective in absorbing these antibodies.

Finally, the carrier (HSA) alone did not affect the levels of these antibodies. This fact provides further support for the specificity of these antibodies. Inhibition of these antibodies with mold antigens and mycotoxins, with the simultaneous presence of IgG, IgA, or IgM antibodies against different molds and mycotoxins (Figs. 1–4), lead us to conclude that these specific antibodies could be used only in a subpopulation of patients or in epidemiologic investigations of mold and mycotoxin exposure. In this regard, our finding of a significant percentage of mold-exposed patients with normal levels of antibodies, as well as a few controls with abnormal levels of mold antibodies, warrants further investigation. In future studies, the issues related to antigenic load, duration of exposure, genetic variabilities, and protective vs. pathogenic antibodies should be addressed.

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