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Health and immunology study following exposure to toxigenic fungi (*Stachybotrys chartarum*) in a water-damaged office environment

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Abstract There is growing concern about adverse health effects of fungal bio-aerosols on occupants of water-damaged buildings. Accidental, occupational exposure in a nonagricultural setting has not been investigated using modern immunological laboratory tests. The objective of this study was to evaluate the health status of office workers after exposure to fungal bio-aerosols, especially *Stachybotrys chartarum* (*atra*) (*S. chartarum*) and its toxigenic metabolites (satratoxins), and to study laboratory parameters or biomarkers related to allergic or toxic human health effects. Exposure characterization and quantification were performed using microscopic, culture, and chemical techniques. The study population ($n = 53$) consisted of 39 female and 14 male employees (mean age 34.8 years) who had worked for a mean of 3.1 years at a problem office site; a control group comprised 21 persons (mean age 37.5 years) without contact with the problem office site. Health complaints were surveyed with a 187-item standardized questionnaire. A comprehensive test battery was used to study the red and white blood cell system, serum chemistry, immunology/antibodies, lymphocyte enumeration and function. Widespread fungal contamination of water-damaged, primarily cellulose material with *S. chartarum* was found. *S. chartarum* produced a macrocyclic trichothecene, satratoxin H, and spirocyclic lactones. Strong associations with exposure indicators and significant differences between employees ($n = 53$) and controls ($n = 21$) were found for lower respiratory system symptoms, dermatological symptoms, eye symptoms, constitutional symptoms, chronic fatigue symptoms and several enumeration and function laboratory tests, mainly of the white blood cell system. The proportion of mature T-lymphocyte cells (CD3%) was lower in employees than in controls, and regression analyses showed significantly lower CD3% among those reporting a history of upper respiratory infections. Specific *S. chartarum* antibody tests (IgE and IgG) showed small differences (NS). It is concluded that prolonged and intense exposure to toxigenic *S. chartarum* and other atypical fungi was associated with reported disorders of the respiratory and central nervous systems, reported disorders of the mucous membranes and a few parameters pertaining to the cellular and humoral immune system, suggesting a possible immune competency dysfunction.

Key words Fungal bio-aerosols · Mycotoxins (satratoxins) · Epidemiology · Immunology · *Stachybotrys chartarum* (*atra*)

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Introduction

There is increasing concern about the health effects of bio-aerosols on occupants of water-damaged buildings which have accumulated high levels of fungal growth. Although associated health complaints are frequently caused by allergic and inflammatory responses, some cases appear to result from intoxication caused by the chemicals produced naturally by the fungi (Hendry and

Cole 1993; Jarvis 1990). These chemicals, so-called mycotoxins, pose a serious health hazard for humans and animals, but until now they have mostly been reported in agricultural settings. The growth of mycotoxin-producing (toxigenic) fungi in indoor air environments has been considered uncommon, but there are some fungi that appear to pose significant health hazards to occupants. One such fungus is *Stachybotrys chartarum* (or *atra*), which produces a series of potent toxins (including satratoxins) (Jarvis 1991) and a variety of compounds affecting the immune system (Jarvis et al. 1995).

Presence of allergenic and toxigenic fungi has previously been reported in damp homes and offices and has been felt to be related to sick building syndrome and cases of building-related diseases (Croft et al. 1986; Dales et al. 1991; Smith et al. 1992; Smoragiewicz et al. 1993; Ruotsalainen et al. 1995); however, systematic studies of health effects and immunological changes related to indoor exposure to trichothecene and other mycotoxins have not been reported. This study was conducted to investigate a sentinel health event and to study possible biomarkers to assess (a) exposure, (b) adverse health reactions of the host, and (c) allergic, inflammatory, or toxic laboratory parameters.

Several employees of a metropolitan, below-street-level office presented to an occupational and environmental specialty clinic with unusual health complaints (Johanning et al. 1993a). Subsequent hygiene investigations of the converted exhibition and basement-level office building in New York City revealed that after recurrent flooding at the subbasement level, extensive fungal contamination of building materials (Sheetrock and insulation material), stored paper products (books), carpets, and the air ventilation system had developed over a period of a few years. Biological sampling revealed an unusual indoor fungus, *Stachybotrys chartarum* (*S. chartarum*), besides predominantly *Penicillium* and *Aspergillus* spp. Chemical analyses of a bulk sample of *S. chartarum*-contaminated Sheetrock revealed the presence of a macrocyclic trichothecene, satratoxin H (Jarvis et al. 1982), which is a potent protein synthesis inhibitor. Chemical compounds of trichothecenes have been reported to cause immunosuppression or alterations (Corrier 1991). Cases of human and animal toxicosis after ingestion of *S. chartarum*-contaminated foodstuffs have been reported sporadically (Ueno 1983; Hintikka 1987). Immune-toxicological laboratory data and clinical knowledge about human cases especially related to inhalation risk are very sparse and incomplete (WHO 1990; Sorenson 1990; Miller 1992). Bio-aerosol exposure effects from toxigenic *S. chartarum* have previously not been studied using modern immunochemistry and flow cytometry.

Materials and methods

Environmental exposure evaluation

Microbial sampling and analyses

Microbial sampling and indoor aerosol fungal identification was conducted shortly before this clinical investigation and before and after bio-remediation efforts were undertaken using sampling methods for viable and nonviable fungi. Quiescent and aggressive airsamples were collected using an Anderson sampler and Burkhard trap. Malt extract agar (MEA), MEA with 20% sucrose (xerophilic fungi), and cellulose Czapek medium (*S. chartarum*) were utilized in subsequent air sampling tests after *S. chartarum* had been identified in bulk samples to allow for selective growth of fungal species. Indoor air sampling results were compared with the outdoor results. Bulk samples were analyzed by dilution culture and microscopically by a leading U.S. specialty laboratory (Burge Laboratory, Harvard University), which also analyzed all air samples.

Air sampling was done quiescently, i.e., under normal operating conditions, and "aggressively", by abnormally disturbing interior finishes to aerosolize settled dust, simulating high indoor activity, and determining whether unusual kinds of fungi were present in dust reservoirs. Bulk samples of moldy interior finishes were also collected and analyzed for the concentrations and kinds of fungi present; this was mostly done at the subbasement level, where the flooding had occurred and widespread fungal growth was visible to the naked eye.

Description of mycotoxin analysis

Two bulk samples (approx. 30 cm^2) of water-damaged, macroscopically "sooty-blackened"-looking, stained Sheetrock paper material taken from the primary source, the subbasement office area (flooded area with widespread visible fungal contamination of various materials) was chemically analyzed after microscopic confirmation of the presence of *S. chartarum*. Black material (*S. chartarum*) was scraped from the largest sample to give 60 mg of powdery black material which was extracted with 2 ml of 20% methanol in chloroform at 40°C, under sonication for 30 min. The extract was passed through a short column of silica gel (1.5 g Whatman LPS-1), washing the column with 10 ml of 8% methanol in dichloromethane. The eluent was removed under a stream of dry nitrogen and the remaining oily material was taken up in 0.5 ml of ethanol and analyzed by reversed phase high-performance liquid chromatography (HPLC: C-18 Rainin 5-µm column 4.6 × 250 mm with a 12-min gradient of 60% –75% methanol in water, flow of 1 ml per minute, and monitoring at 260 nm). A peak of 9.5 min corresponded in retention time to that of *satratoxin H*. The total amount of satratoxin H in the 60-mg sample was estimated to be ca. 1 µg.

Under different HPLC conditions, several additional mycotoxins of the immunosuppressant *phenylspirodrames* class were detected. These HPLC conditions were: C-18 Supelco 5-µm column 4.6 × 250 mm with a solvent system of 5% acetic acid in water (solvent A) and methanol (solvent B), at a flow rate of 1.2 ml/min; 60% B from $t = 0$ to $t = 3$ min, then 60% – 75% B from $t = 3$ to $t = 18$ min. Under these conditions, the phenylspirodrames have retention times (RTs) ranging from 11 to 16 min. The two major phenylspirodrames detected (at levels of ca. 5 – 10 µg/60-mg sample) were *Stachybotrylactone* and *Stachybotrylactone acetate* (RT = 13.0 and 15.1 min, respectively). Under these conditions, satratoxin H has a retention time of 5.5 min. Analysis of this extract on normal phase HPLC on both silica and PEI silica (both with and without coinjections of standard compounds) (Jarvis et al. 1995) confirmed the peak assignments made by reversed phase HPLC.

Epidemiology and serological tests

Health complaints and medical, occupational and environmental history were surveyed with a 187-item standardized questionnaire compiled from questionnaires of the Occupational Safety and Health Administration (OSHA), the Scandinavian Indoor Climate/Work Environment and the National Institute for Occupational Safety and Health (NIOSH). The study population (employees with more than 3 months' employment) was compared with a convenience control group with similar job activities (office workers). The control group was selected to be closely matched by important confounders from the same metropolitan area, assuming similar residential and outdoor mold exposure. The study population ($n = 53$) consisted of 39 female and 14 male employees with a mean age of 34.8 years and a mean employment of 3.1 years (40 h/week) at the problem office site; ten of these persons were active smokers. The control group ($n = 21$) consisted of 11 females and ten males, with a mean age of 37.5 years and four active smokers, who had no contact with the problem office site (exposure duration = 0). Additional exposure indicators within the study population included: duration of employment at problem office; visible moldy stains on wall and ceiling material; participation in the water flood cleanup in subbasement areas; floor level of office location (verified exposure differences).

Cumulative symptom complex scores by major target organs and constitutional factors were formed to estimate severity of health effects. Symptom complexes included "upper respiratory problems" (nasal irritation, burning, stuffiness and congestion, sore and burning throat), "lower respiratory problems" (recurrent shortness of breath, cough, chest tightness, wheezing), "central nervous system" (CNS) (severe headaches, concentration problems, irritability, dizziness or lightheadedness, sleeping problems, extreme mental fatigue), "eye" (burning, irritation, blurry vision), "constitutional" [unexplained, low-grade fever, tender/swollen lymph nodes, flu-like symptoms (myalgia)] and "skin" (burning, erythematous rash, hair loss). Study participants were also questioned about the occurrence and frequency of infections requiring medical attention such as "upper respiratory infections" (URI), "flu" (respiratory), "gynecological and other yeast infections" (yeast), "urinary or bladder infection" (UTI), occurring in the yeast prior to the study and during the period of employment at the problem building. Participants under medical treatment for related problems and using medication, i.e., antibiotics or anti-inflammatory medication, were excluded from the study.

Laboratory analyses of blood samples

A comprehensive immunological test battery developed by NIOSH immunochemistry research section in Cincinnati, Ohio to detect immunomodulation from exposure to xenobiotics was used to study the red and white blood cell systems, serum chemistry, immune function and immunoglobulin antibodies. The selection of the laboratory parameters was based on a review of reported in vitro and in vivo effects from exposure to allergenic fungi and mycotoxins. Peripheral venous blood was collected and handled in a routine manner and immediately shipped at ambient temperature to NIOSH, Cincinnati. Tests performed on fresh blood or isolated cells were initiated by the laboratory within 14–16 h.

Flow cytometry

Whole-blood samples were stained with two- and three-color monoclonal antibodies (Coulter Cytometry, Hialeah, Fl.) : Mo2-RD1/KC56-FITC (CD14/CD45); IgG1-RD1/IgG1-FITC; T8-FITC/NKH-1-RD1 (CD8/CD56); T11-RD1/B4-FITC (CD2/CD19); IgG2b-ECD/IgG1-RD1/IgG1-RD1 (isotype control); CD3-ECD/T4-RD1/T8-RD1 (CD3/CD4/CD8); IgG1-RD1/IgG2a-FITC (isotype control) and CD3-RD1/I3-FITC (CD3/HLA-DR). After incubation following the manufacturer's directions, the samples were lysed by an automatic whole-blood lysis technique (Multi-Qprep, Coulter). Samples were analyzed on a flow cytometer with an automatic carousal loader (EPICS XI with MCL, Coulter). Laser alignment (DNA Check, Coulter) and fluorescence standardization (Standard-Brite, Coulter) were performed using latex beads. Electronic compensation for spectral overlap of the fluorochromes was performed using freshly drawn historical control blood samples stained with CD3-ECD/T4-RD1/T8-RD1. The 488-nm line from the argon laser was used to excite the fluorescein isothiocynate (FITC), phycoerythrin (PE), and electronically coupled dyes (ECD). Forward-versus side-light scatter histograms were collected and a back gating paradigm was used to select $CD45_{bright}/CD14_{dim}$ stained cells, yielding a lymphocyte gate. Five thousand mononuclear cells were analyzed for each subject's sample. Total lymphocyte subset counts were calculated by multiplying the percent positive by the total number of lymphocytes. Complete blood counts with differentials were performed using a laser hemocytometer (STKS, Coulter).

Lymphocyte function tests

Peripheral blood mononuclear cells isolated for lymphocyte proliferation assays by means of Ficoll-Hypaque density gradient centrifugation. The cells were resuspended in complete medium [CM; RPMI 1640 + 100 units/ml penicillin with 100 µg/ml streptomycin and 2 mm L-glutamine (GIBCO Laboratories, Grand Island, N.Y.)] with 10% FCS, at a concentration of 1×10^6 viable cells per ml (by trypan blue exclusion. One hundred-microliter aliquots of the cell suspensions were dispensed into appropriate wells of 96-well plates to yield 1×10^5 cells/well in individual wells (cellular additions were performed in triplicate for all dilutions). The following mitogens were diluted in CM and added at the final concentrations specified: phytohemagglutinin (PHA, Difco, Detroit, Mich.), 50 µg/ml, 5 µg/ml, and 0.5 µg/ml; concanavalin A (ConA, Sigma Chemical Co., St. Louis, Mo.) 20 µg/ml, 2 µg/ml, and 0.2 µg/ml; pokeweed mitogen (PWM, Sigma) 1 µg/ml, 0.1 µg/ml, and 0.01 µg/ml. The first three wells in each row received media in place of mitogen and served as a nonstimulated background control. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Incubation times were 72 h (PHA) or 120 h (ConA and PWM). The wells were pulsed with 50 µl, of ^{3}H-thymidine (ICN, Costa Mesa, Calif.; specific activity, 2–10 Ci/mmol), prepared as a fresh stock of 20 µCi/ml diluted in CM. After 4–6 h, the cells were harvested on a cell harvester and placed into minivials with 2 ml scintillation cocktail. Disintegration per minute (cpm) of triplicate samples were counted on a liquid scintillation counter.

Determination of natural killer (NK) cell activity

One hundred-microliter aliquots of peripheral blood mononuclear cells isolated as described above were dispensed into appropriate wells of 96-well round-bottomed tissue culture plates with covers to yield 1×10^6, 5×10^5, and 2.5×10^5 cells/well in individual wells (cellular additions were performed in triplicate for all dilutions). This yielded 100:1, 50:1, and 25:1 effector target cell ratios as explained further below. The target cells (K 562) were grown as stationary suspension culture in CM. They were labelled with 250–400 µCi of ^{51}Cr (as sodium chromate. NEN Research Products, Boston, Mass.) per 10^7 cells. The cells were labelled during their exponential growth phase (24–28 h) after transfer. Target cells were pelleted ($200 \times g$ for 10 min) and the supernatant was aspirated. Aqueous $Na_2\ ^{51}CrO_4$ was added and the volume of the cells adjusted to 1 ml of CM. The cells were incubated for 1 h at 37 °C in 5% CO_2. Cells were washed

and pelleted twice more and resuspended as a suspension. The labelled cells were dispensed at 100 µl (10^4 cells) into appropriate wells of 96-well culture plates containing NK cells, from above, and incubated for 4 h at 37 °C. After incubation supernatants were removed and counted in a gamma counter (COBRA, Packard Instruments, Downers Grove, Ill.). The percentage of maximal ^{51}Cr release was calculated by the following formula:

$$\frac{\text{cpm experimental release} - \text{cpm spontaneous release} \times 100}{\text{cpm maximal release (10\% Triton X)} - \text{cpm spontaneous release}}$$

Mold-specific antibody tests

Mold-specific antibody tests were done by the IBT reference laboratory (Kansas, Miss.). For fungal-specific IgE, fungal extracts (*Penicillium notatum*, *Aspergillus versicolor*, and *S. chartarum*) were coupled to paper discs by the diazo method and were used to test sera by the modified RAST protocol (Leary and Halsey 1984). For fungal-specific IgG measurements, a microtiter-based EIA was used with the same antigens as above. Each serum was assayed at two different dilutions in duplicate. Fungal extracts were obtained from Miles Allergy Products, Biopol, and Allergon AB. Total IgE was measured using the RIST technique.

Statistical analysis

All data were computed, tabulated and analyzed using parametric (ANOVA, logistical and linear regression) and non-parametric tests [chi-square, Kruskall-Wallis (K–W)] with a statistical PC package (SPSS for windows). The K–W test was used to compare means if there was heterogeneity of variance. The Mantel–Haenszel test for linear association was used to identify significant trends across building exposure categories. Statistical significance levels were set at $P \leq 0.05$. Variables that were positively skewed were log-transformed for analysis.

Participation in the study was voluntary and confidentiality was assured. The study protocol was approved by the institutional review board. Because several employees had been noted to have health problems related in time and place to work activity in the contaminated office building, two extensive bio-remediation attempts were made and administrative control measures were in place to limit exposure to *S. chartarum* materials approximately 18 and 3 months before this investigation was conducted in the fall of 1993 (Johanning et al. 1993b).

Results

Exposure sampling results

Microscopic and culture analyses showed widespread fungal contamination primarily in the offices, library, and storage area on the subbasement level (lowest floor level where repeated water flooding had occurred) and to a lesser extent in the above basement area offices. In the subbasement office area on walls, water-damaged Sheetrock (drywall) facing paper, stored books and catalogs, and floor carpeting, widespread and high concentrations of *S. chartarum*, a fungus not typically found in occupied indoor environments, were identified. Microscopic presence of *S. chartarum*, besides *Cladosporium*, *Penicillium*, and *Aspergillus*, was found in a bulk sample of the internal insulation of the ventilation ducts from the basement air handling unit (HVAC). By the design of the HVAC system, the "return air" from the contaminated subbasement space used as an air plenum was vented to the above basement office level. Other predominant indoor species were *Aspergillus versicolor*, *A. niger*, *Penicillium* spp., and *Cladosporium*.

Concentrations of *Stachybotrys* on moldy gypsum board and moldy books all taken from the subbasement level approached 10^6 colony-forming units (cfu) per cm^{-2}. *Penicillium* or *Aspergillus* species (*A. versicolor*, *A. glaucus*, and *A. sydowi*) dominated or co-dominated 11 of 24 air samples (range 10–163 cfu/m^3). Air samples collected outdoors were dominated by *Cladosporium* or *A. niger*. *Stachybotrys* and *Aspergillus* species such as *A. versicolor*, *A. glaucus*, and *A. sydowi* were not found in outdoor air samples.

Quiescent and aggressive air samples collected on MEA plus 20% sucrose medium often contained *Penicillium* and *Aspergillus* species (maximum concentration exceeding 3×10^5 cfu/m^3 in two aggressive samples).

Stachybotrys was found in five of the 21 air samples collected on cellulose Czapek medium. In two air samples ("aggressive" sampling in the subbasement) the concentration of *Stachybotrys* was approximately 2×10^4 cfu/m^3, which suggests that very strong reservoirs of this kind of spore existed at the time of sampling (sampling done at the time of this investigation). Results of aerosol sampling prior to this study are shown in Table 1.

The indoor dry bulb temperature ranged from 24.4° to 28.1°C and the relative humidity (%RH) from 52.6 to 58.3 (outdoor 28.1°C; 55%RH) in June; in November the respective figures were 22°–25°C and 34–39%RH (outdoor 19°C; 43%RH). Water leaks from adjacent building walls were detectable on the subbasement floor level, contributing to visible water saturation of drywall material. Particulate concentrations collected under quiescent conditions ranged from 338 080 to 1 012 340 particles per cubit foot (particles/ft^3), compared to an outdoor concentration of 752 630 particles/ft^3. Carbon dioxide concentrations indoors ranged from 500 to 650 ppm. A strong "musty" odor in the basement and subbasement, which was stronger on rainy days or when outdoor humidity was high, was noted by the investigator and building occupants during the initial inspection.

Based on an assessment of all sampling results, visible inspection and available best evidence information, the fungal exposure categories shown in Table 2 were formed (outside sampling results considered as "typical" and "normal" reference).

Table 1 Sampling results in respect to airborne fungi collected on cellulose Czapek agar by location. Sampling was conducted during a midsummer month, using an SAS air sampler operating at a flow rate of 0.2 cubic meters of air per minute

Location	Quiescent sampling			Aggressive sampling		
	Concentration (cfu/m^3)	Taxa	(%)	Concentration (cfu/m^3)	Taxa	(%)
Outdoor	58	*Cladosporium*	45			
		Alternaria	27			
		Penicillium	18			
		A. fumigatus	9			
Ground floor	48	*Cladosporium*	56	937	*Cladosporium*	69
		Aspergillus spp.	22		Nonsporulating	8
		Paecilomyces	11		*Ulocladium*	8
		Penicillium	11		*Alternaria*	5
					Aspergillus spp.	2
					Coelomycetes	2
					Epicoccum	2
Basement	106	*Cladosporium*	53	603	*Cladosporium*	74
		Aspergillus spp.	21		*Alternaria*	9
		Penicillium	16		Non-sporulating	9
		Alternaria	11		*Aspergillus* spp.	3
					Coelomycetes	3
					Stachybotrys	3
Basement	79	*Cladosporium*	60	302	*Stachybotrys*	28
		Aspergillus spp.	13		*Aspergillus* spp.	28
		Penicillium	13		*Chaetomium*	11
		Alternaria	7		Nonsporulating	11
		Curvularia	7		*Cladosporium*	6
					Paecilomyces	6
					Penicillium	6
					Ulocladium	6
Subbasement	58	*Cladosporium*	45	> 20000 overgrown	*Stachybotrys*	97
		Alternaria	9		*Fusarium*	2
		Aspergillus spp.	9		*Chaetomium*	1
		Coelomycetes	9			
		Yeast	18			
Subbasement	116	*Cladosporium*	48	> 20000 overgrown	*Stachybotrys*	100
		Penicillium	14		*Alternaria*	
		Stachybotrys	14		*Cladosporium*	
		Alternaria	10		Nonsporulating	
		Trichoderma	5			
		Ulocladium	5			

Table 2 Fungal exposure categories (see text for details)

Office location	Exposure cattegory	Description
Ground floor	None/low	No *Stachybotrys* fungi identified and no/minimal atypical fungal exposure.
Basement	Moderate	*Stachybotrys* found in ventilation duct material (bulk sample) and a few air samples; moderate atypical fungal exposure.
Subbasement	High	Extensive *Stachybotrys* contamination (bulk and air samples positive) and moderate atypical exposure to other fungi.

Health effects and laboratory tests

Strong associations with exposure indicators among employees and significant differences between employees and controls were found for important health outcomes and several laboratory tests. As compared with controls, greater proportion of employees in the contaminated office building had a history of infections and symptoms in all major organ systems during the past year (Table 3). Differences between groups were statistically significant for lower respiratory system, dermatological, eye, constitutional, and chronic fatigue symptoms. Indoor fungal exposure indicators such as "subbasement office location" (Table 3) participation in the "cleanup activity" of the flood damage (subbasement), and visible "moldy stains in office" (data not shown) were associated with reports of disorders of several major organ systems (upper respiratory system,

Table 3 Health effect survey responses of workers exposed to toxigenic fungi compared wtih controls and by workplace location (% of responses). The internal comparison group excludes four subjects who had no specific floor locations (*N/D* = not determined)

Organ system affected	By external comparison:		By internal comparison, according to office location		
	Controls ($n = 21$)	Subjects ($n = 53$) P^a	Ground floor ($n = 7$) : no/low fungal exposure	Basement ($n = 33$): moderate fungal exposure	Subbasement ($n = 9$) P^b P^c(trend) high fungal exposure
Respiratory system					
Upper airways	43	57	14	67	56 *
Lower airways	43	76**	43	79	78
Worse in past year	5	13	0	12	22
Skin	19	47*	14	52	67 *
Central nervous system	52	70	43	70	89
Eyes	19	57**	0	64	67 * *
Constitutional (feverish, adenopathy, flu-like)	5	28*	14	21	56
"Multiple chemical hypersensitivity"	33	43	14	42	56
Chronic fatigue symptoms	5	24*	0	21	67 * *
Allergy history	48	60	43	64	78
Infection (within past year)					
Upper respiratory infections	47	62	43	70	56
Yeast	14	26	0	30	44
Urinary tract infections	5	15	0	21	0
Health worse since employment at problem-building	N/D	51	0	67	88 * *

*$P < 0.05$
**$P < 0.01$
[a] Difference between external controls and subjects (chi-square)
[b] Difference between subjects (internal controls) by office location (chi-square)
[c] Trend by Mantel-Haenszel test for linear association

skin, eyes), chronic fatigue symptoms, and "health worse since employment in the contaminated building" (Table 3). Duration of employment (mean 3.17 years, SD 2.5) was associated with upper respiratory tract ($r = 0.27$, $P < .001$), skin ($r = 0.24$, $P < 0.08$), and CNS disorders ($r = 0.27$, $P < 0.054$).

White blood cell (WBC) analysis showed few differences between subjects and controls, or by exposure location (Table 4a). However, comparing office locations and controls showed the highest WBC levels among basement employees (Table 5). The eosinophil fraction (%) was marginally higher ($P = 0.06$) among all employees versus controls (Table 4a), and the eosinophil number was marginally higher among basement employees ($P = 0.09$).

Lymphocyte enumeration analyses showed few differences between subjects and controls. However, employees had a significantly ($P = 0.01$) lower proportion of mature lymphocytes [CD3 in % (pan T cell antigen receptor)] than controls (Table 4b), although only non significant differences were observed between office locations (Table 5). NK cell % ($P = 0.054$) and number ($P = 0.03$) were highest among basement employees (Table 5).

Substantial, but non significant, differences were observed for total immunoglobulin E (IgE) count and C-reactive protein and immunoglobulin G (IgG) (Table 4c).

Only two employees, who had been working on the basement and subbasement level for more than 1 year – both with an atopic history – had a positive IgE test (RAST) for *S. chartarum* (Table 4d). Subjects and controls had low mean IgG antibody levels to *S. chartarum* with small differences (Table 4c). Lymphocyte function tests (ConA and PHA mitogen proliferation) were slightly (but not significantly) lower for subbasement versus basement and ground floor employees (Fig. 1). However, this tendency was not observed for PWM. Subbasement and basement employees had consistently lower values than controls for all lymphocyte function tests (but the differences were not significant; data not shown). Separating out basement office workers with a history of yeast infections did not substantially alter the previous results.

Multivariate analyses of health outcomes by selected immunological laboratory tests and for possible confounders (gender, smoking, allergy history and mold problems at home) showed CD3% and PWM proliferation test scores to be significantly lower among those with a history of URI (Table 6). History of previous recurrent fungal/yeast infections was associated with a lessened response to the ConA mitogen proliferation test (primarily CD8 T-lymphocytes). Number of upper respiratory symptoms was associated with lower IgG antibodies to *S. chartarum* (*S. chartarum* IgG). Other

Table 4 White blood cell, lymphocyte and immunoglobulin enumeration tests for workers exposed to toxigenic fungi compared with controls and by workplace location

	By external comparison:				By internal comparison, according to office location:		
	Controls ($n = 21$)		Subjects ($n = 53$)		Ground floor[a] ($n = 7$)	Basement[b] ($n = 33$)	Subbasement[c] ($n = 9$)
	Mean	SD	Mean	SD	Mean	Mean	Mean
a) WBC (1000 cells/μl):	6.1	1.3	6	1.6	5.1	6.3	6.37
Lymphocyte%	31	6	30.5	5.5	31.8	30.3	29.3
Monocyte%	7.8	1.7	8.4	3.4	11.5	7.8	8.4
Neutrophil%	58	7.6	57.5	7.5	53	58.1	58.3
Eosinophil%	2.2	1.3	3	2.2	3	3.2	3.2
Basophil%	0.7	0.4	0.7	0.4	0.8	0.7	0.6
b) Lymphocyte subset (%):							
CD3 (mature T cell)	75.7	15.3	73.5*	6.7	72.4	74.1	71.9
CD4 (helper cell)	47.4	11.6	46.7	8	48.1	46.1	47.2
CD8 (suppressor cell)	25.7	9.3	24.3	7	21	25.8	21.8
CD4/CDB	2.1	2.2	1.6	1.3	1.1	1.79	1.51
CD19 (early B cell)	10.5	4.3	11.7	5.3	12.1	11.6	11.8
CD56 (NK cell)	2.4	3.2	2.8	2.4	1.7	3	3.3
c) Immunoglobulin (mg/dl):							
IgA	190	86	182	78	187	185	151
IgG	1008	216	981	214	1082	957	938
IgM	146	81	148	76	160	138	179
IgG-PNC	42	44	31	20	28	31	38
IgG-ASP	50	63	40	32	40	39	47
IgG-Stachy.	9	10	7	6	8	7	9
CRP	14	17	7.2	1	6.9	7.4	6.9
IgE	76	79	171	303	112	221	91
d) Immunoglobulin E (absolute number/subjects)							
IgE (> 100 IU/l)	6		22		4	14	3
IgE-Penicillium	0		5		0	5	0
IgE-Aspergillus	0		1		0	1	0
IgE-Stachybotrys	0		2		0	1	1

*$P = 0.01$ (K-W)
[a] No/low fungal exposure
[b] Moderate fungal exposure
[c] High fungal exposure (see Table 2)

associations included chronic fatigue symptoms with lower total IgE (data not shown).

Among control variables, a history of preexisting allergy problems (atopic conditions, previous positive allergy tests, family history of allergy) was significantly associated with a higher number of lower respiratory system symptoms. Compared with men, women had far more yeast infections, upper respiratory health complaints, and worse health since at exposure location. Eighty-six percent of basement and subbasement employees were female, compared to 29% of ground floor employees and 52% of controls.

Discussion

In this epidemiological and immunochemistry laboratory study of exposure to toxigenic *S. chartarum* and other fungal species, we found significantly increased proportions of office workers with health problems typical for a "sick building" compared with a population of similar demographic profile and occupation. We also found an association of fungal exposure with abnormalities of the cellular and humoral immune system, though the magnitude of some differences was small. Self-reported exposure indicators and office areas with highest documented contamination were associated with abnormal findings of some components of the immune response system, primarily some nonspecific (eosinophils, NK cells, and C-reactive protein) and specific adaptive cells (T and B lymphocytes). Higher and longer indoor exposure to atypical fungi and *S. chartarum* or its chemical products appears to be associated with increased immune reactivity and possibly impaired immunity. While it is possible that some significant association occurred due to chance (type 1 error), some significant association between exposure

Table 5 Significant immune competence laboratory parameters compared with controls and by work location (*A* ANOVA, *K – W* Kruskal–Wallis)

Lab. test	Controls ($n = 21$)	Ground floor ($n = 7$)	Basements (includes subbasement) ($n = 42$)	*P*	Statistical test
WBC(k/μl)	6.06	5.05	6.29	0.024	A
CD3% (T Lymphocytes)	75.66	72.9	73.65	0.04	KW
NK # (CD 56)	41.83	23.59	55.45	0.03	A

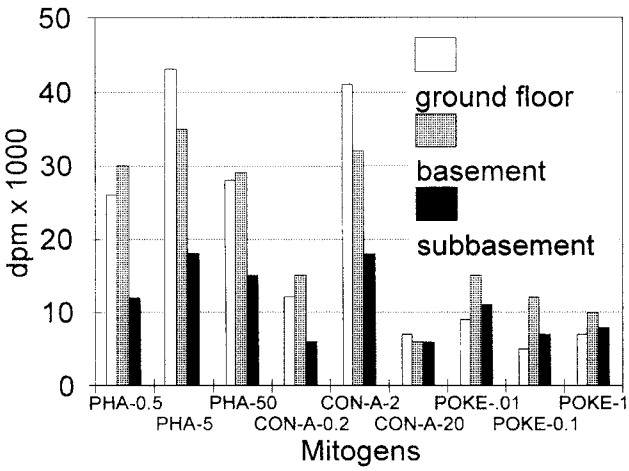

Fig. 1 lymphocyte function test (mitogen proliferation) by office location

measures and laboratory tests may not have been observed because of low statistical power due to the small sample size.

Inflammatory responses and T or B lymphocyte effects following fungal toxin (i.e., trichothecene) exposure have been shown in laboratory studies, but there are currently no human studies available for comparison of our findings (Ueno 1983; Corrier 1991). The benefit of such investigations compared with some controlled laboratory studies is that immunological interactions of all contributing components of the body's defense system are taking place and impaired immune surveillance and suppression outcome can be studied in a more realistic setting. However, the exposure conditions and dose of all office workers cannot be assumed to be similar and of equal range. After adverse health effects had been previously diagnosed in several office workers, environmental control measures and several, although not successful, remediation and administrative control attempts were made before this investigation. This may have influenced our clinical and laboratory findings. Since the laboratory values are labile and dependent, in part, on exposure to environmental allergens and toxins, reduction of exposure should result in return of laboratory values to normal ranges over a period of several weeks to months. This results in a weakening of associations between some exposure measures and laboratory results. The stronger associations between exposure and health symptoms are likely due, in part, to the fact that subjects were asked about health symptoms and illnesses occurring over the course of the past year, when exposure was presumably greater, and not simply current symptoms.

Sampling of aerosolized *Stachybotrys* is difficult and frequently may result in false-negatives if selective growth media and adequate sampling methods are not used. *Stachybotrys* grows well on cellulose media, but can easily be overgrown on other media by *Aspergillus* or *Penicillium* spp. Also, spores of *S. chartarum* are typically very slimy and do not easily become airborne. In most reported air sampling of other investigations, selective cellulose Czapek media has not been used and may thus underrepresent true airborne levels of viable *S. chartarum* spores. Reliable sampling methods for airborne mycotoxins in field studies are not readily available and were not used in this study. Therefore we could not quantify levels of airborne mycotoxins. Results of the chemical analyses of bulk samples under laboratory conditions may not be representative of the field conditions and aerosolized mycotoxin concentration apparently depends on several environmental conditions, such as temperature, humidity (water activity), draft, vibration, and physical disturbance of material. Furthermore, culture measurement of airborne viable *Stachybotrys* spores may underrepresent true exposure, since nonviable spores, which under dry conditions may easily get airborne, typically contain a very high concentration of mycotoxins (Sorenson 1990).

Marked leukopenia or acute "radiation-mimetic" effects on the blood cell system with subsequent sepsis-like opportunistic infections after trichothecene ingestion were not found as described in clinical reports of earlier cases in Eastern Europe (Jarmai 1929; Sarkisov 1947; Ozegovics 1971). In these cases probably higher toxin concentrations were present and described laboratory changes of the red and white blood cell system lasted only a few days to weeks in the subjects who survived the intoxications. We found a nonsignificant trend toward an increased unusual occurrence and frequency of infections (URI, fungal and yeast, UTI), compared with a population of similar demographic profile.

Lymphocytes have receptors for mitogen and can respond to these without prior sensitization. This can be used in nonspecific laboratory function tests. PHA and ConA respond more with T cells, and PWM is a T cell-dependent stimulator of B lymphocytes.

Table 6 Association of health outcomes with selected immunological laboratory tests ($n = 74$) : odds ratios and b-coefficients (adjusted for gender, smoking, allergy history, and mold noticed at home). Upper and lower respiratory symptom score computed by linear regression; other health outcomes computed by logistic regression. (*URI* upper respiratory infection, *YEAST INF.* systemic or local fungal infection, UTI urinary tract infection, *N/D* no subjects had risk factor present).

Laboratory test	URI		Yeast inf.		UTI		Health[c]		No. of Upper resp. symptoms		No. of Lower resp. symptoms	
	Yes: $n = 43$	P^b	Yes: $n = 17$	P^b	Yes: $n = 9$	P^b	Yes: $n = 27$	P^b	b-coefficient	P^b	b-coefficient	P^b
CD3 % (T lymph.)	0.93	0.03	0.97		0.95		0.92		− 0.006		− 0.006	
ConA1[a]	0.8		0.5	0.02	0.7		0.66		− 0.068		− 0.146	
ConA5	0.99		0.95	0.01	0.98		0.98		− 0.005		− 0.009	
ConA25[a]	0.81		0.4	0.02	0.61		0.89		− 0.125		0.055	
$PWM_C^\circ 04$[a]	0.65	0.05	0.93		1.31		0.63		− 0.023		0.107	
$PWM_L^\circ 2$[a]	0.61	0.03	0.73		1.3		0.66		0.005		0.061	
PWM 1[a]	0.6	0.04	0.85		1.24		0.6		− 0.013		0.139	
IgG[a]	0.96		5.05		3.29		0.29		0.685		0.113	
STA IgG[a]	0.64		0.85		0.77		0.71		− 0.188	0.04	0.025	
PNC IgG[a]	0.98		1.73		1.53		1.41		0.006		0.235	
ASP IgG[a]	0.93		1.57		1.99		1.45		− 0.083		0.115	
IgE Total[a]	1.01		0.88		1.19		0.76		0.066		0.156	
STA IgE ($n = 2$)	N/D		N/D		N/D		1.18		0.662		0.965	
PNC IgE ($n = 5$)	2.46		5.78		4.58		1.07		0.295		0.963	
ASP IgE ($n = 1$)	N/D		N/D		0.68		N/D		1.424		1.058	
Female	0.74		7.89		N/D		13.13	0.005	− 0.408	0.04	0.166	
Smoke	0.77		0.54		0		3.26		− 0.383		− 0.916	
Mold noticed at home	0.7		1.35		0.52		1.84		0.050		0.258	
Allergy History	2.48		3.31		0.96		3.37		0.353		1.064	0.002

[b] Only $P < 0.05$ listed
[a] Log transformed
[c] Health condition worse since employment at exposure location

Inability to proliferate is a possible sign of impaired immunity (Rowlands et al. 1991).

Immune modulation and suppression of the humoral and cellular cell system after mycotoxin exposure have been studied in vivo and in vitro in animal and laboratory experiments. There may be a synergistic effect of several different mycotoxins after inhalation of fungal material in an indoor setting such as we investigated (Moss and Frank 1987). Other typical environmental factors identified in previous "sick building syndrome" cases, such as dust, carbon monoxide, carbon dioxide, work organizational factors, temperature, and climate control problems, may also have influenced our findings; however, we have no indication for this.

Some *Penicillium* and *Aspergillus* species found in damp houses have been shown to have toxic effects in cell line tests with a mean mortality of up to 50%; *Aspergillus* samples were less cytotoxic than *Penicillium* (Smith et al. 1992). However, metabolites from *S. chartarum* appear to be more potent. Trichothecene mycotoxins (*S. chartarum*) are considered the most potent small molecule inhibitor of protein synthesis through inhibition of the peptidyl transferase activity (McLaughlin et al. 1977; Ueno 1983). These toxins can cause alveolar macrophage defects and affect phagocytosis. They have been investigated for use in cancer treatment (Goodwin et al. 1978) but also in chemical-biological warfare. Presence of fungal chemical metabolites has been reported in several cases of animal and human ingestion-related mycotoxicosis (Hintikka 1987; Jarvis 1990), Mycotoxins, such as satratoxin H of the trichothecene group, have been shown to cause depressed T or B lymphocyte activity, suppressed immunoglobulin and antibody production, reduced complement or interferon activity, and impaired macrophage-effector cell function (Corrier 1991). Impaired migration-chemotaxis and phagocytosis of human neutrophils have also been reported (Yarom et al. 1984). Weakened resistance to infections of salmonella, tuberculosis, listeria, herpes simplex, candida and cryptococcus has been demonstrated after trichothecene poisoning in various animal models. It may also suppress the tumor defense mechanism and weaken the host control of tumor cell growth.

Currently the IARC classifies aflatoxin, a toxin from *Aspergillus* species, with "sufficient evidence for human and animal carcinogenicity" (liver), but trichothecene toxins (T-2 toxin, Fusarien toxins) with

"limited evidence" for animals and "inadequate evidence" (no data available) for humans (IARC 1993). Satratoxin H has not been classified. Some epidemiological studies have shown a higher rate of upper respiratory tract and lung cancer in workers in the mining, grain, and food handling industries, who have a high fungal product inhalation risk (Kuzák et al. 1970; Sorenson 1990).

Specific fungal antibodies have been used as possible mold spore exposure markers in the working environment (Eduard 1993); however, in this study we did not find that higher immunoglobulin G and E antibodies to *S. chartarum* were statistically associated with studied health outcomes. It is possible that the fungal antigens used in the laboratory analysis were not identical with fungal antigens from the problem building, that the original strands had only a low antigenicity, or that the tested subjects had an insufficient exposure to develop a measurable antibody immune reaction. Also, continued antigen exposure in the office environment (since remediation efforts were not fully successful in eliminating the indoor *S. chartarum* source) might have increased the formation of IgG_4 subclass complexes (not measured in our screening tests), resulting in higher removal (turnover) and lack of serological elevation of IgG *S. chartarum* (Van der Zee et al. 1989; Spielberg 1989). Valid reference ranges for *S. chartarum* IgG antibodies have currently not been firmly established.

Changes in immunoglobulins (IgG, IgA, IgM, and IgE) similar to those detected by us were reported in a Russian study of workers handling mycotoxin-contaminated foodstuffs, primarily deoxynivalenol [vomitoxin (trichothecene), considered the least acute toxic trichothecene (IARC 1993)] (Tutelyan et al. 1992). Increase in IgA production (contrary to our study) and IgA nephropathy and decrease in IgG and IgM (as in our study) after ingestion of vomitoxin was reported in a mice experiment (Pestka et al. 1989). Renal failure and IgG deposition in the glomeruli after inhalation of ochratoxin produced by *Aspergillus ochraceus* was found in a farmer (Paolo et al. 1993).

The production of toxic chemical metabolites from *S. chartarum* apparently depends on several environmental factors, such as temperature (2–40°C) and water content of the fungal-contaminated material (80–100% RH). High cellulose content material like wall paper, paper-coated gypsum board (Sheetrock), straw, and books combined with very high water saturation has been shown to promote the growth and presence of macrocyclic trichothecenes, mainly satratoxin G and H, with adverse biological activity (Nikulin et al. 1994). Particularly in the subbasement area of the investigated building (with offices, library, storage, the ventilation/climate control unit, and return air plenum space), similar environmental conditions existed for some time periods, which made toxigenic spore production highly likely, although we could not test and confirm airborne mycotoxin contamination with generally accepted and established sampling methods.

Few and sparse clinical descriptions of human cases of toxicosis from *S. chartarum* are available. It was thought that mainly farmworkers and laboratory personnel (employed in military and food safety research) belong to the high-risk group for mycotoxin-related diseases. However, one recently published case report about an airborne outbreak of *S. chartarum* toxicosis related illnesses in a family home to the effects of trichothecene chemicals (Croft et al. 1986). In this case study no serological tests, biomarkers, or immunological parameters were reported. Unusual indoor air contamination with *S. chartarum* mycotoxins was thought to be responsible for several confirmed cases of extreme chronic fatigue syndrome in hospital workers (Auger et al. 1994). Indoor air *Stachybotrys* exposure has been found to be associated with an exacerbation of asthma (Flannigan et al. 1991; Kozak et al. 1979).

Pathogenic effects, even fatal, of *S. chartarum* or its chemical derivatives were described in animals in the 1920s and 1940s by Russian, German, and Hungarian veterinary researchers (e.g., Jarmai 1929). In the United States the first detailed English-language account of such cases and of his own work was reported by Forgacs in 1972. The early medical case descriptions, mostly by veterinarians, included: severe skin and mucous membrane irritation (burning), bleeding disorders, conjunctivitis, upper and lower respiratory disorders, low-grade fever, and cardiac arrhythmia. A German group suggested a treatment protocol for acute and fulminant trichothecene poisoning based on animal experiments, finding positive effects from use of steroids and activated charcoal combined with general supportive care (Forth et al. 1990).

Fungal aerosol mixtures have been associated with allergic rhinitis, allergic asthma, and hypersensitivity pneumonitis (Pope et al. 1993). However, there is now growing evidence of direct cytotoxic and neurotoxic effects from microbial aerosols. Gram-negative bacterial cell products, such as endotoxin and β-1, 3-glucan, may be responsible for respiratory inflammatory disorders and organic dust toxic syndrome (Rylander and Fogelmark 1994; Rylander and Jacobs 1994). Bacterial aerosol exposure was not considered relevant in this investigation, based on low concentrations in earlier tests and microbiological environmental assessment (data not shown). Typically high-level bacterial exposures can be found in chicken and swine confinement facilities and farming situations.

The possible role of fungi and previously investigated mycotoxins in indoor air situations and health implications have been reviewed (Hendry and Cole 1993; Miller 1992). Pulmonary exposure to toxic fungi in occupational settings such as the food processing industry or laboratories was found to be associated with infections, allergy, or hypersensitivity (Sorenson et al. 1987). A WHO Task Group concluded that an association

between trichothecene exposure and human disease episodes is possible; however, only limited data are available (WHO 1990). This paucity of past data may have been related to the lack of the immunochemistry tests now available to clinicians and to inadequate exposure assessment. Immunotoxicological effects principally depend on the exposure dose and timing. As mentioned earlier, some immunological effects may be only transient and of short duration and difficult to detect using routine medical tests. Individuals may also show variable susceptibility.

Flow cytometry analysis, now available in many clinical settings, may aid in the diagnosis of immunopathology, although alterations may not be specific or clinically important in the diagnosis of infectious diseases (Rowlands et al. 1991; Keren et al. 1994). Presented laboratory tests combined with clinical-epidemiological studies and adequate exposure assessments appear to be helpful in identifying building occupants who may benefit from medical and administrative intervention. Environmental control measures should include removal of building material with dust restriction techniques similar to modern asbestos abatement methods to prevent unnecessary exposure risk to building occupants and remediation workers (NYC-DOH 1993; Johanning et al. 1993b).

In conclusion, self-reported health status indicator changes and lower T-lymphocyte proportions and dysfunction as well as some other immunochemistry alterations were associated with onset, intensity, and duration of occupational exposure to toxigenic *S. chartarum* combined with other atypical fungi. Modern immunochemistry panels, which include lymphocyte enumeration and function tests, may aid in the clinical investigation of subjects with serious adverse health effects related to intense fungal bio-aerosol exposure. Long-term risk and clinical prognosis of toxigenic fungal inhalation, particularly of *S. chartarum* and its mycotoxins, in humans is not well known. Further studies are required to test the validity of biomarkers of exposure and disease related to fungal aerosol inhalation and to evaluate long-term health consequences.

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