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Production of Proinflammatory Mediators by Indoor Air Bacteria and Fungal Spores in Mouse and Human Cell Lines

Kati Huttunen, Anne Hyvärinen, Aino Nevalainen, Hannu Komulainen, and Maija-Riitta Hirvonen

Department of Environmental Health, National Public Health Institute, Kuopio, Finland

We compared the inflammatory and cytotoxic responses caused by household mold and bacteria in human and mouse cell lines. We studied the fungi *Aspergillus versicolor*, *Penicillium spinulosum*, and *Stachybotrys chartarum* and the bacteria *Bacillus cereus*, *Pseudomonas fluorescens*, and *Streptomyces californicus* for their cytotoxicity and ability to stimulate the production of inflammatory mediators in mouse RAW264.7 and human 28SC macrophage cell lines and in the human A549 lung epithelial cell line in 24-hr exposure to 10^5 , 10^6 , and 10^7 microbes/mL. We studied time dependency by terminating the exposure to 10^6 microbes/mL after 3, 6, 12, 24, and 48 hr. We analyzed production of the cytokines tumor necrosis factor- α and interleukins 6 and 1β (TNF- α , IL-6, IL- 1β , respectively) and measured nitric oxide production using the Griess method, expression of inducible NO-synthase with Western Blot analysis, and cytotoxicity with the MTT-test. All bacteria strongly induced the production of TNF- α , IL-6 and, to a lesser extent, the formation of IL- 1β in mouse macrophages. Only the spores of *Str. californicus* induced the production of NO and IL-6 in both human and mouse cells. In contrast, exposure to fungal strains did not markedly increase the production of NO or any cytokine in the studied cell lines except for *Sta. chartarum*, which increased IL-6 production somewhat in human lung epithelial cells. These microbes were less cytotoxic to human cells than to mouse cells. On the basis of equivalent numbers of bacteria and spores of fungi added to cell cultures, the overall potency to stimulate the production of proinflammatory mediators decreased in the order *Ps. fluorescens* > *Str. californicus* > *B. cereus* > *Sta. chartarum* > *A. versicolor* > *P. spinulosum*. These data suggest that bacteria in water-damaged buildings should also be considered as causative agents of adverse inflammatory effects. **Key words:** bacteria, cytokine production, fungi, inflammation, mold. *Environ Health Perspect* 111:85–92 (2003). [Online 4 December 2002] doi:10.1289/ehp.5478 available via <http://dx.doi.org/>

Exposure to microbes is recognized as a potential cause of inflammation-related health problems among occupants of moldy buildings (Husman 1996; Peat et al. 1998). Many different microbes, including a variety of fungi and bacteria, thrive in the conditions offered by moist building materials, which contain both the nutrients and the moisture needed for microbial growth (Hyvärinen et al. 2002; Murtoniemi et al. 2001; Nikulin et al. 1994). The current understanding is that microbial growth affects indoor air quality, and occupants are exposed to biological pollutants, which may lead to adverse health effects. Currently it is not known which components of the microbial flora are most harmful to occupants of moldy buildings. Comparisons of the different microbes present in such environments are required to evaluate the potential health effects of a given microbe. Our working hypothesis is that bacteria isolated from moldy buildings are also important in causing inflammatory and cytotoxic responses.

Indoor bacterial flora include both gram-positive and gram-negative strains, which grow in moist building materials (Andersson et al. 1997; Hyvärinen et al. 2002). The gram-positive sporulating bacteria *Streptomyces* spp. have been frequently

isolated from water-damaged buildings, but they are not part of the normal microbial flora of indoor air (Nevalainen et al. 1991). The presence of *Streptomyces* is also an indicator of moisture damage in buildings (Samson et al. 1994). We demonstrated previously that the spores of streptomycetes originating from moldy buildings can evoke intense production of inflammatory mediators both in mouse and human cell lines (Hirvonen et al. 1997; Jussila et al. 1999) *in vitro* and in mouse lungs *in vivo* (Jussila et al. 2001). Thus, the correlation found between *in vitro* and *in vivo* data strongly supports the view that streptomycetes play a role in the cascade of events leading to adverse health effects in occupants of moldy buildings. Moreover, these data suggest that phagocytes and epithelial cells may be the target cells producing these inflammatory mediators in the lung.

The gram-positive bacteria *Bacillus* spp. are common environmental bacteria and are also found in indoor environments. Recently, *Bacillus cereus* has been isolated from damaged building materials, and also toxin-producing strains of *Bacillus* spp. have been identified (Andersson et al. 1999). However, the ability of these strains to cause inflammatory responses has not been shown. In contrast to gram-positive bacteria, all gram-negative

bacteria contain lipopolysaccharide (LPS) as a component of their cell membrane. LPS has profound stimulating effects on immunological cells, inducing the production of cytokines and nitric oxide (Raetz et al. 1991). Among the gram-negative bacteria, the genus *Pseudomonas* spp. is found in many natural sources, including soils, waters, and outdoor air. *Ps. fluorescens* is the most common *Pseudomonas* species in the outdoor air (Nevalainen 1989).

Penicillium and *Aspergillus* spp. are frequently detected at higher concentrations in moldy buildings, although these microbes are common airborne fungi in indoor environments (Flannigan and Morey 1996; Hyvärinen et al. 1993; Nevalainen et al. 1991). Both these genera include also mycotoxin-producing species (Frisvad and Gravesen 1994), and thus exposure to these species can potentially lead to adverse health effects. However, the conditions supporting the toxin production and the mechanisms of exposure and the health effects have yet to be identified. *Penicillium spinulosum* is considered a generally nontoxic species, whereas *Aspergillus versicolor* is both a common toxin producer and an indicator of mold problems in a building (Samson et al. 1994). *Stachybotrys chartarum*, a well-known producer of potent toxins (Gravesen et al. 1994), prefers a substrate with a high moisture content, thus indicating severe moisture damage when isolated from buildings (Johanning et al. 1996). The growth of this fungus in water-damaged buildings has been associated with adverse health effects such as respiratory and other symptoms (Gordon et al. 1999; Johanning et al. 1996, 1999). Moreover, *Sta. chartarum* has been associated with a cluster of cases of idiopathic pulmonary hemosiderosis (IPH) in infants in North America and Europe (Centers for Disease Control and Prevention 1994; Flappan et al. 1999).

Address correspondence to K. Huttunen, Department of Environmental Health, National Public Health Institute, PO Box 95, FIN-70701 Kuopio, Finland. Telephone: +358 17 201320. Fax: +358 17 201265. E-mail: Kati.Huttunen@ktl.fi

We thank H. Martikainen and L. Heikkinen for their excellent technical assistance. This study was supported by the Academy of Finland, the Finnish Research Programme on Environmental Health (SYTTY) and The Juha Vainio Foundation.

Received 18 January 2002; accepted 18 June 2002.

Our aim in this study was to compare the cytotoxic and inflammatory potential of the fungal and bacterial strains considered important in indoor environments. We selected *Aspergillus versicolor* and *Stachybotrys chartarum* because of their characteristic occurrence in moldy indoor environments and because of their importance as potential toxin producers. *Penicillium spinulosum* was selected to represent common indoor air fungi because it is present in all types of indoor environments. We selected the gram-positive bacterial strain *Streptomyces californicus* because of its characteristic occurrence in water-damaged buildings and its proposed inflammatory potential. We selected *Bacillus cereus* to represent another gram-positive strain and *Pseudomonas fluorescens* to represent a gram-negative strain; both are common environmental bacteria present in indoor environments. We also studied the differences in the ability of the bacteria and fungi to trigger production of nitric oxide and the proinflammatory cytokines tumor necrosis factor- α (TNF- α) and interleukins 1 β and 6 (IL-1 β and IL-6, respectively) in human and mouse macrophages as well as in human lung epithelial cells. In addition, we evaluated the cytotoxicity of exposure to these microbes.

Materials and Methods

Microbes. In this study we used three fungal strains, *Aspergillus versicolor*, *Penicillium spinulosum*, and *Stachybotrys chartarum*, and three bacterial strains, *Bacillus cereus*, *Pseudomonas fluorescens*, and *Streptomyces californicus*. The strains of *Aspergillus versicolor* and *Penicillium spinulosum* were isolated from indoor air of buildings with moisture problems, and *Sta. chartarum* was isolated from a damaged building material sample. *B. cereus* and *Ps. fluorescens* were isolated from indoor air of residences without proven water damage, and *Str. californicus* was isolated from a building with moisture problems. Microbes were sampled from indoor air using a six-stage impactor and 2% malt extract agar (MEA; Biokar Diagnostics, Beauvais, France) for fungi and tryptone yeast-glucose agar (TYG; Bacto Plate Count Agar, Difco Laboratories,

Detroit, MI, USA) for bacteria. *Sta. chartarum* was isolated on malt extract agar.

The identification of the fungal strains was confirmed by the CBS identification service (Centraal Bureau van Schimmelcultures, Utrecht, the Netherlands) and *Str. californicus* was confirmed by the DSM identification service (DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). The strains of *B. cereus* and *Ps. fluorescens* were identified at the National Veterinary and Food Research Institute, Kuopio Regional Laboratory, Finland. The microbial strains were stored at -20°C until the experiments. The fungal strains were cultured on 2% MEA and bacterial strains on TYG as a dense culture and incubated in the dark at 25°C for 7 days and 20°C for 5 days, respectively. Spores and cells were collected with a sterile loop and suspended in 5 mL of Hank's Balanced Salt Solution (HBSS) containing 0.0001% Triton X-100. We determined the spore concentration of *Str. californicus* using an epifluorescence microscope, and concentrations of other spores or cells were counted in a Bürker chamber under light microscope.

Cell culture. A mouse macrophage cell line (RAW264.7), a human macrophage cell line (28SC), and a human lung epithelial cell line (A549) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). All the cell types were cultured at 37°C in a 5% CO_2 atmosphere in cell line-specific culture medium.

Mouse RAW264.7 macrophages were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin (all from Gibco BRL, Paisley, UK). The human 28SC macrophages (hematopoietic cell line) were cultured in Iscove's modified Dulbecco's medium supplemented with 10% heat-inactivated FBS, 0.05 mM 2-mercaptoethanol, 0.03 mM thymidine, and 0.01 mM hypoxanthine. Human alveolar type II epithelial-like cell line A549 was cultured in Ham's F-12K Medium supplemented with 10% heat-inactivated FBS. Cells (5×10^5

cells/mL) were dispensed to six-well plates, 2 mL/well.

We primed the human cell lines with interferon- γ (10 ng/mL) and added antimicrobial agents (nystatin and penicillin-streptomycin) after the dispensing of the cells. The cells were allowed to adhere for 24 hr and fresh complete medium was added before exposure. The human macrophage cells grow in suspension and hence were exposed without changing the culture medium, also 24 hr after the dispensation.

In the dose-response study, the macrophages and epithelial cells were exposed to three doses (10^5 , 10^6 , and 10^7 microbes/mL) of each microbe for 24 hr. For the time-course study, all three cell lines were exposed to all six microbial strains at the dose of 10^6 microbes/mL, and the exposure was terminated at five time points after the start of exposure (3, 6, 12, 24, and 48 hr). The dose used in time course study was chosen on the basis of the responses seen in dose-response study. We used bacterial LPS as a positive control at the dose of 10 $\mu\text{g/mL}$. After the exposure, the adherent cells were resuspended in the culture medium either by scraping (RAW264.7) or trypsin incubation (A549), and the cell suspension was centrifuged (5 min, 8000 rpm) to separate the cells from the culture medium. The supernatants were stored at -80°C for the analyses of cytokines, and the cells were frozen immediately in dry ice and stored at -80°C for Western blot analysis.

Nitrite analysis. We measured nitric oxide spectrophotometrically as the stable metabolite, nitrite (NO_2) according to the Griess method (Green et al. 1982). Briefly, Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% phosphoric acid) was mixed 1:1 with samples of the cell culture medium. Nitrite forms a colored chromophore with the reagent, with an absorbance maximum at wavelength of 543 nm, which was measured with an enzyme-linked immunosorbent assay (ELISA) microplate reader (iEMS Reader MF; Labsystems, Turku, Finland). We quantified the production of nitrite by comparing the results with absorbances of standard

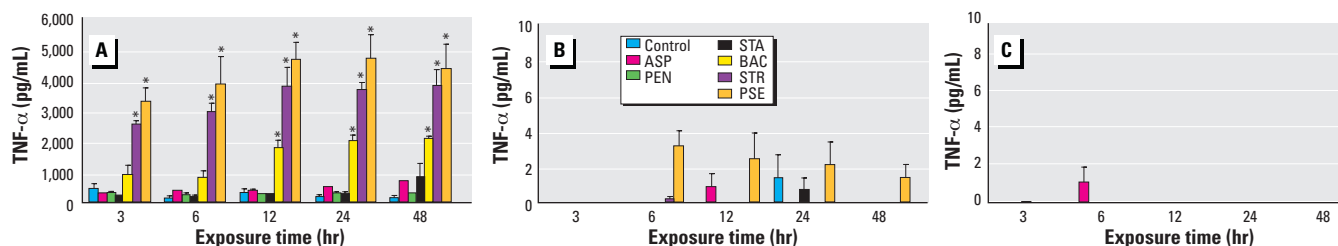


Figure 1. The time frame of TNF- α production after exposure to a single dose (10^6 microbes/mL) of *A. versicolor* (ASP), *P. spinulosum* (PEN), *Sta. chartarum* (STA), *B. cereus* (BAC), *Str. californicus* (STR), and *Ps. fluorescens* (PSE) in (A) mouse RAW264.7 macrophages, (B) human 28SC cells, and (C) human A549 lung epithelial cells. Each column represents mean $\pm$ SEM of three independent experiments done in duplicate.

*Statistically significant difference from the respective control, $p < 0.05$.

solutions of sodium nitrite. Samples of three independent experiments were analyzed in duplicate.

Western blot analysis. We analyzed expression of the inducible nitric oxide synthase (iNOS) with the Western blot technique. The frozen cells were lysed in lysis buffer [20 mM TrisHCl, 2 mM EDTA, 3% (v/v) Triton X-100, 100 mM NaCl] by pulling the cell suspension through a 26-gauge needle and incubating the suspension for 30 min on ice. Afterward the cell fragments were centrifuged (13,000 rpm) for 10 min. Sample buffer (1.5 mM bromophenol blue; 0.14 M SDS; 0.12 M TrisHCl; 0.1 M β -mercaptoethanol; 1.3 M glycerol) was heated and added 1:2 to the supernatant, which was subsequently boiled at 95°C for 6 min. The samples were subjected to electrophoresis through 7.5% Tris-glycine gel (Criterion gels; Bio-Rad Laboratories, Hercules, CA, USA) to differentiate proteins by size. From the gel, proteins were transferred electrophoretically in a transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol, 0.05% SDS) to a polyvinylidene difluoride membrane.

Membranes were incubated overnight at 4°C in blocking buffer [100 mM TrisHCl; 150 mM NaCl; 50 g/l bovine serum albumin (BSA)], rinsed with washing buffer (10 mM TrisHCl; 150 mM NaCl; 0.1 % Tween; pH 7.4), and incubated in primary antibody solution [0.1% rabbit anti-iNOS (Transduction Laboratories, Lexington, KY, USA)] in washing buffer for 2 hr at room temperature. After

the primary incubation, we washed (6 × 5 min) the membranes with washing buffer and incubated them in a second antibody solution (0.05% alkaline phosphatase conjugated goat anti-rabbit IgG (Zymax Zymed, South San Francisco, CA, USA) in washing buffer) for 1 hr. The membranes were washed again, bathed in developing buffer (100 mM NaCl; 100 mM Tris; 50 mM MgCl₂ × 6 H₂O) and developed in developing solution [330 mg/L nitro blue tetrazolium (NBT); 165 mg/L bromochloroindonyl phosphate disodium (BCIP; both from Sigma Chemical Company, St. Louis, MO, USA) in developing buffer]. Colored bands 130 kD in size were visually detected from the membrane.

Cytokine analysis. We analyzed TNF- α , IL-6, and IL-1 β from the cell culture medium immunochemically using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA), as described earlier (Ruotsalainen et al. 1998). Samples were processed according to the manufacturer's protocol and analyzed with an ELISA microplate reader by comparing the absorbances of the samples to the standard curve. We analyzed samples of three independent experiments in duplicate.

Cell viability. We determined the viability of the macrophages by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) test to detect living mitochondria (Mosmann 1983). Live mitochondria can transform MTT (Sigma) to formazan, which can be measured with a spectrophotometer. We compared the proportion of viable cells in exposed samples to a

control sample. The presence of microbes among the exposed cells does not interfere with the MTT test at tested concentrations. We estimated the viability of the cells in the control samples after staining the cells with trypan blue solution. Samples of three independent experiments were analyzed in duplicate.

Statistical analysis. We analyzed the data using analysis of variance (ANOVA) and least squares difference test to compare exposed group to control group (SPSS version 7.51; SPSS Inc., Chicago, IL, USA). The difference was considered to be statistically significant at $p < 0.05$. The trend in each dose response was tested with the nonparametric Jonckheere-Terpstra test, which tests whether k independent samples defined by a grouping variable are from the same population (SPSS, version 10.1.3, SPSS Inc.). The trend was considered to be statistically significant at $p < 0.05$.

Results

Cytokine production. TNF- α . Mouse RAW264.7 macrophages produced TNF- α in a dose- and time-dependent manner after the exposure to the bacterial strains *B. cereus* (test for trend $p = 0.004$) and *Str. californicus* ($p = 0.003$). *Ps. fluorescens* ($p = 0.088$) and the positive control LPS also caused intense TNF- α production (Table 1, Figure 1). Both the gram-negative bacterium *Ps. fluorescens* and the spores of the gram-positive bacterium *Str. californicus* induced a strong increase in TNF- α production in these cells, whereas *B. cereus* caused a clear but lower response than the other bacteria (Table 1). The time course of this response revealed that TNF- α production was induced in 3 hr, and the maximum was obtained in 12 hr (Figure 1). The time courses were similar for all bacteria. In contrast, even the highest dose of the fungal spores induced only minor TNF- α production in RAW264.7 cells, with *Sta. chartarum* being the most potent (Table 1). However, the trend for dose response was evident in all three fungal strains, with p -values ranging from 0.004 for *A. versicolor* and *Ps. fluorescens* to 0.02 for *Sta. chartarum*.

Human 28SC macrophages produced significant amounts of TNF- α only after the exposure to LPS. This peaked at 3 hr (up to 90 ± 60 pg/mL) and gradually declined during the time course of the experiment. *Ps. fluorescens* caused a slight increase in the production of TNF- α , whereas the other microbes did not stimulate the human 28SC macrophages to produce TNF- α (Table 2, Figure 1). Neither LPS nor any of the tested fungi or bacteria triggered TNF- α production in human A549 epithelial cell line (Figure 1, Table 3).

Interleukin-6. Mouse RAW264.7 macrophages produced IL-6 in a dose- and time-dependent manner after exposure to all three

Table 1. Dose response of the production of inflammatory mediators and cell viability (mean $\pm$ SEM) of mouse RAW264.7 macrophages after 24-hr exposure to six microbes.

Exposure agent	Dose (mL)	NO (μ M)	Cell viability (% of control)	IL-1 β (pg/mL)	TNF- α (pg/mL)	IL-6 (pg/mL)
C	—	1.0 $\pm$ 0.4	100	BD	94 $\pm$ 14	3 $\pm$ 1
LPS	10 μ g/mL	33.7 $\pm$ 0.5*	19 $\pm$ 5*	330 $\pm$ 80*	6,500 $\pm$ 1,300*	2,180 $\pm$ 30*
Fungi						
ASP	10 ⁵	0.8 $\pm$ 0.2	96 $\pm$ 6	BD	140 $\pm$ 13	BD
	10 ⁶	0.7 $\pm$ 0.2	61 $\pm$ 6*	BD	445 $\pm$ 27	BD
	10 ⁷	0.9 $\pm$ 0.1	13 $\pm$ 2*	BD	470 $\pm$ 100	BD
PEN	10 ⁵	1.0 $\pm$ 0.3	99 $\pm$ 7	BD	86 $\pm$ 11	BD
	10 ⁶	0.7 $\pm$ 0.1	74 $\pm$ 2*	BD	190 $\pm$ 30	BD
	10 ⁷	0.7 $\pm$ 0.1	43 $\pm$ 2*	BD	1,900 $\pm$ 450	2 $\pm$ 1
STA	10 ⁵	0.7 $\pm$ 0.2	116 $\pm$ 2	BD	270 $\pm$ 21	BD
	10 ⁶	1.2 $\pm$ 0.2	26 $\pm$ 8*	BD	740 $\pm$ 240	8 $\pm$ 4
	10 ⁷	NM	NM	NM	NM	NM
Bacteria						
BAC	10 ⁵	1.2 $\pm$ 0.2	108 $\pm$ 7	BD	880 $\pm$ 260	6 $\pm$ 2
	10 ⁶	1.8 $\pm$ 0.4	71 $\pm$ 10*	7 $\pm$ 5	3,900 $\pm$ 700*	110 $\pm$ 50
	10 ⁷	1.5 $\pm$ 0.2	26 $\pm$ 4*	140 $\pm$ 70	3,100 $\pm$ 830	1,400 $\pm$ 340*
STRE	10 ⁵	2.9 $\pm$ 0.8	83 $\pm$ 4*	BD	3,600 $\pm$ 610*	30 $\pm$ 8
	10 ⁶	20.5 $\pm$ 2.7*	40 $\pm$ 4*	15 $\pm$ 2	5,300 $\pm$ 750*	2,100 $\pm$ 40*
	10 ⁷	28.3 $\pm$ 1.6*	24 $\pm$ 2*	60 $\pm$ 23	6,100 $\pm$ 880*	2,150 $\pm$ 40*
PSE	10 ⁵	28.6 $\pm$ 2.5*	40 $\pm$ 6*	180 $\pm$ 60	7,100 $\pm$ 920*	2,270 $\pm$ 50*
	10 ⁶	28.6 $\pm$ 1.6*	30 $\pm$ 3*	300 $\pm$ 120	8,000 $\pm$ 1100*	2,370 $\pm$ 20*
	10 ⁷	4.6 $\pm$ 1.0*	44 $\pm$ 10*	230 $\pm$ 60	6,900 $\pm$ 800*	2,370 $\pm$ 30*

Abbreviations: ASP, *Aspergillus versicolor*; BAC, *Bacillus cereus*; BD, below detection limit; C, buffer control; NM, not measured; PEN, *Penicillium spinulosum*; PSE, *Pseudomonas fluorescens*; STA, *Stachybotrys chartarum*; STRE, *Streptomyces californicus*.

*Statistically significant difference compared to buffer control, $p < 0.05$.

bacterial species (*B. cereus*, *Str. californicus*, *Ps. fluorescens*; Table 1, Figure 2). The *p*-values for dose response were 0.000, 0.001, and 0.016, respectively. The positive control LPS also caused an intense IL-6 production (Table 1). Among the bacterial strains, the gram-negative *Ps. fluorescens* was the most potent. The spores of *Str. californicus* also triggered a strong overall response in the mouse macrophages, whereas

the induction of IL-6 by *B. cereus* was clearly weaker (Table 1). The increase in the IL-6 production was detected at 3 hr of exposure, and it reached the maximum in 6 hr with *Ps. fluorescens*, whereas *Str. californicus* displayed a slower time-course (Figure 2). The fungal strains *A. versicolor*, *P. spinulosum*, and *Sta. chartarum* did not cause IL-6 production in mouse macrophages (Table 1).

Table 2. Dose response of the production of inflammatory mediators and cell viability (mean $\pm$ SEM) of human 28SC macrophages after 24-hr exposure to six microbes.

Exposure agent	Dose (mL)	NO (μ M)	Cell viability (% of control)	IL-1 β (pg/mL)	TNF- α (pg/mL)	IL-6 (pg/mL)
C	—	1.5 $\pm$ 0.2	100	BD	BD	2 $\pm$ 1
LPS	10 μ g/mL	2.1 $\pm$ 0.2	82 $\pm$ 6*	BD	95 $\pm$ 19*	670 $\pm$ 10*
Fungi						
ASP	10 ⁵	1.9 $\pm$ 0.1	94 $\pm$ 6	BD	BD	2 $\pm$ 1
	10 ⁶	1.8 $\pm$ 0.3	81 $\pm$ 3*	BD	BD	2 $\pm$ 1
	10 ⁷	1.7 $\pm$ 0.3	53 $\pm$ 6*	BD	BD	3 $\pm$ 1
PEN	10 ⁵	2.0 $\pm$ 0.2	92 $\pm$ 8	BD	BD	2 $\pm$ 0.2
	10 ⁶	1.5 $\pm$ 0.2	78 $\pm$ 6*	BD	BD	1 $\pm$ 1
	10 ⁷	1.5 $\pm$ 0.2	69 $\pm$ 6*	BD	BD	2 $\pm$ 0.3
STA	10 ⁵	1.5 $\pm$ 0.1	95 $\pm$ 6	BD	BD	1 $\pm$ 0.1
	10 ⁶	1.9 $\pm$ 0.2	36 $\pm$ 4*	BD	BD	3 $\pm$ 1
	10 ⁷	NM	NM	NM	NM	NM
Bacteria						
BAC	10 ⁵	2.1 $\pm$ 0.3	101 $\pm$ 4	BD	BD	2 $\pm$ 1
	10 ⁶	1.7 $\pm$ 0.3	97 $\pm$ 4	BD	BD	2 $\pm$ 1
	10 ⁷	1.8 $\pm$ 0.3	75 $\pm$ 6*	BD	BD	2 $\pm$ 0.3
STRE	10 ⁵	1.6 $\pm$ 0.2	97 $\pm$ 9	BD	BD	4 $\pm$ 1
	10 ⁶	1.5 $\pm$ 0.2	95 $\pm$ 8	BD	BD	14 $\pm$ 4
	10 ⁷	2.1 $\pm$ 0.1	72 $\pm$ 7*	BD	6 $\pm$ 2	170 $\pm$ 70*
PSE	10 ⁵	1.7 $\pm$ 0.2	95 $\pm$ 7	BD	BD	30 $\pm$ 20
	10 ⁶	1.5 $\pm$ 0.2	100 $\pm$ 7	BD	5 $\pm$ 2	340 $\pm$ 180*
	10 ⁷	1.7 $\pm$ 0.2	79 $\pm$ 15	BD	18 $\pm$ 4	480 $\pm$ 180*

Abbreviations: ASP, *Aspergillus versicolor*; BAC, *Bacillus cereus*; BD, below detection limit; C, buffer control; NM, not measured; PEN, *Penicillium spinulosum*; PSE, *Pseudomonas fluorescens*; STA, *Stachybotrys chartarum*; STRE, *Streptomyces californicus*.

*Statistically significant difference compared to buffer control, *p* < 0.05.

Table 3. Dose response of the production of inflammatory mediators and cell viability (mean $\pm$ SEM) of human A549 lung epithelial cells after 24-hr exposure to six microbes.

Exposure agent	Dose (mL)	NO (μ M)	Cell viability (% of control)	IL-1 β (pg/mL)	TNF- α (pg/mL)	IL-6 (pg/mL)
C	—	0.9 $\pm$ 0.2	100	BD	BD	6 $\pm$ 0.6
LPS	10 μ g/mL	1.2 $\pm$ 0.2	90 $\pm$ 10	BD	BD	110 $\pm$ 61*
Fungi						
ASP	10 ⁵	0.9 $\pm$ 0.3	103 $\pm$ 5	BD	BD	6 $\pm$ 0.5
	10 ⁶	0.9 $\pm$ 0.2	78 $\pm$ 5*	BD	BD	6 $\pm$ 0.5
	10 ⁷	0.9 $\pm$ 0.2	49 $\pm$ 4*	BD	BD	17 $\pm$ 4.5
PEN	10 ⁵	1.5 $\pm$ 0.3	116 $\pm$ 10	BD	BD	5 $\pm$ 2.1
	10 ⁶	0.7 $\pm$ 0.3	90 $\pm$ 8	BD	BD	6 $\pm$ 0.8
	10 ⁷	1.1 $\pm$ 0.3	83 $\pm$ 6	BD	BD	14 $\pm$ 2.1
STA	10 ⁵	1.7 $\pm$ 0.4	125 $\pm$ 14	BD	BD	10 $\pm$ 2.4
	10 ⁶	1.1 $\pm$ 0.2	100 $\pm$ 16	BD	BD	22 $\pm$ 6.5
	10 ⁷	4.4 $\pm$ 0.1*	42 $\pm$ 2*	NM	NM	NM
Bacteria						
BAC	10 ⁵	1.0 $\pm$ 0.3	108 $\pm$ 10	BD	BD	7 $\pm$ 0.9
	10 ⁶	1.2 $\pm$ 0.3	103 $\pm$ 3	BD	BD	4 $\pm$ 0.8
	10 ⁷	0.7 $\pm$ 0.1	45 $\pm$ 4*	BD	BD	17 $\pm$ 4.8
STRE	10 ⁵	0.9 $\pm$ 0.3	151 $\pm$ 12	BD	BD	16 $\pm$ 1.2
	10 ⁶	0.7 $\pm$ 0.2	176 $\pm$ 18	BD	BD	25 $\pm$ 1.8
	10 ⁷	1.9 $\pm$ 0.3*	199 $\pm$ 16	BD	BD	19 $\pm$ 0.2
PSE	10 ⁵	0.8 $\pm$ 0.2	117 $\pm$ 11	BD	BD	11 $\pm$ 2.0
	10 ⁶	0.8 $\pm$ 0.1	92 $\pm$ 14	BD	BD	37 $\pm$ 11
	10 ⁷	1.1 $\pm$ 0.2	62 $\pm$ 8*	BD	BD	100 $\pm$ 12*

Abbreviations: ASP, *Aspergillus versicolor*; BAC, *Bacillus cereus*; BD, below detection limit; C, buffer control; NM, not measured; PEN, *Penicillium spinulosum*; PSE, *Pseudomonas fluorescens*; STA, *Stachybotrys chartarum*; STRE, *Streptomyces californicus*.

*Statistically significant difference compared to buffer control, *p* < 0.05.

In human macrophages, only *Ps. fluorescens* (test for trend *p* = 0.000), *Str. californicus* (*p* = 0.000), and positive control LPS stimulated a statistically significant IL-6 production, but the detected levels were much lower than those in mouse macrophages (Table 2). The maximum response was attained in 24 hr (Figure 2). Neither spores of *B. cereus* nor any of the fungal strains increased the production of IL-6 in human macrophages at any dose (Table 2) or at any time point (Figure 2). Human epithelial cells produced moderately increased amounts of this cytokine after exposure to *Str. californicus* and *Ps. fluorescens* (*p*-values for dose response, 0.023 and 0.000, respectively), whereas a weaker trend could be seen after exposure to *A. versicolor* (*p* = 0.055) and *P. spinulosum* (*p* = 0.065; Table 3). Fungal strains were thus less potent than bacterial strains. In time-course study, only the spores of *Sta. chartarum* and *Ps. fluorescens* induced slight production of IL-6 in these cells (Figure 2).

Interleukin-1 β . Similar to the other measured cytokines, gram-negative bacterial LPS and *Ps. fluorescens* (test for trend *p* = 0.015) induced mouse RAW264.7 macrophages to produce equally high concentrations of IL-1 β , and the trend for dose response was seen also for *B. cereus* (*p* = 0.012) and *Str. californicus* (*p* = 0.001; Table 1). The maximum was reached at 12 hr, and the levels declined thereafter (Figure 3). In addition, the spores of *Str. californicus* induced slight IL-1 β production with a similar time pattern. The spores of the fungal strains did not increase the production of IL-1 β in mouse macrophages (Table 1, Figure 3). Both human cell lines failed to produce significant amounts of IL-1 β at all (Table 2 and 3, Figure 3).

Nitric oxide production. LPS (not shown) and *Str. californicus* induced increased NO production after 3 hr, and *Ps. fluorescens* after 6 hr of exposure in mouse RAW264.7 macrophages (Figure 4). The gram-negative bacterium *Ps. fluorescens* was again the most potent inducer among the microbes at low doses, whereas at the highest dose (10⁷ cells/mL) the concentration of NO decreased (Table 1, Figure 4). The spores of gram-positive *Str. californicus* elicited almost as high concentrations of NO in cell culture media as *Ps. fluorescens*, but with a clear increasing trend (*p* = 0.000). The other gram-positive bacteria and fungi did not cause any significant increase in NO production in this time frame. Inducible nitric oxide synthase was detectable by Western blotting in mouse cells, which produced increased amounts of NO (Figure 5). The expression was barely detectable at the highest concentration (10⁷ microbes/mL) of *Ps. fluorescens*, in concordance with the low NO concentration in the culture medium (Table 1). This dose might

have caused cytotoxicity so rapidly that there was no time for induction of iNOS.

The spores of *Str. californicus* marginally increased the NO production in human macrophages in the time-course study. The concentrations were much lower than those induced in mouse macrophages. The increase was statistically significant at 6 and 24 hr (Fig 4). Human epithelial cells produced slightly increased amounts of NO only after the exposure to the spores of *Str. californicus* (test for trend $p = 0.075$). In the time-course study the difference compared to control cells was clearest at 24 hr (Table 3, Figure 4). Inducible nitric oxide synthase was not detectable by Western blotting from the human cell lines (data not shown).

Cell viability. Bacteria decreased the viability of mouse macrophages in the same order as they induced the production of proinflammatory cytokines (Table 1). The gram-negative bacterium *Ps. fluorescens* was the most toxic to these cells, followed by the

spores of gram-positive *Str. californicus* and *B. cereus*. With respect to the fungal spores, the cytotoxicity decreased in the order *Sta. chartarum* > *A. versicolor* > *P. spinulosum*, with the highest concentration (10^6 spores/mL for *Sta. chartarum*, 10^7 spores/mL for *A. versicolor* and *P. spinulosum*) clearly decreasing cell viability. The trend for dose response was statistically significant (p -values ranging from 0.000 to 0.005) in all but the case of *Sta. chartarum* ($p = 0.146$), for which the dose of 10^7 spores/mL was not tested. The time-course of the cell viability by 10^6 spores/mL showed that if the viability of the exposed cells was decreased, it took place in 3 hr (Figure 6). At 48 hr some recovery in cell numbers might even have taken place (Figure 6).

In human macrophages only the highest concentration (10^7 microbes/mL) of all of the bacteria caused a 20–30% decrease in cell viability (Table 2). The cytotoxicity of the fungi decreased in the order *Sta. chartarum* > *A. versicolor* > *P. spinulosum*. The trend for dose

response was statistically significant (p -values ranging from 0.000 to 0.019) in all but *Ps. fluorescens* ($p = 0.207$). Cell viability was not notably decreased at the concentration of 10^6 microbes/mL (Figure 6), which was used in all biochemical time-course analyses. In human epithelial cells most microbes were clearly cytotoxic only at the concentration of 10^7 microbes/mL. There was a statistically significant trend for dose response for all microbes (p -values ranging from 0.000 to 0.027), although *Str. californicus* was not cytotoxic in these cells (Table 3).

Discussion

The current experiment reveals major differences between the inflammatory and cytotoxic potency of different groups of microbes present in indoor air. Interestingly, both gram-negative bacteria, *Ps. fluorescens*, and spores of gram-positive bacteria, *Str. californicus*, triggered profound effects in murine and human cells, whereas the spores of the

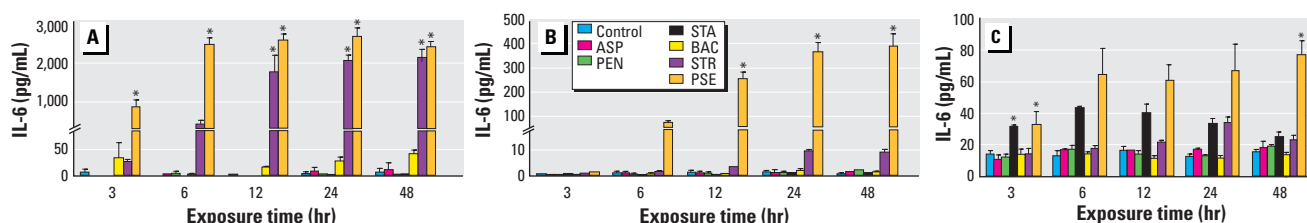


Figure 2. The time frame of IL-6 production after exposure to a single dose (10^6 microbes/mL) of *A. versicolor* (ASP), *P. spinulosum* (PEN), *Sta. chartarum* (STA), *B. cereus* (BAC), *Str. californicus* (STR), and *Ps. fluorescens* (PSE) in (A) mouse RAW264.7 macrophages, (B) human 28SC cells, and (C) human A549 lung epithelial cells. Each column represents mean $\pm$ SEM of three independent experiments done in duplicate.

*Statistically significant difference from control, $p < 0.05$.

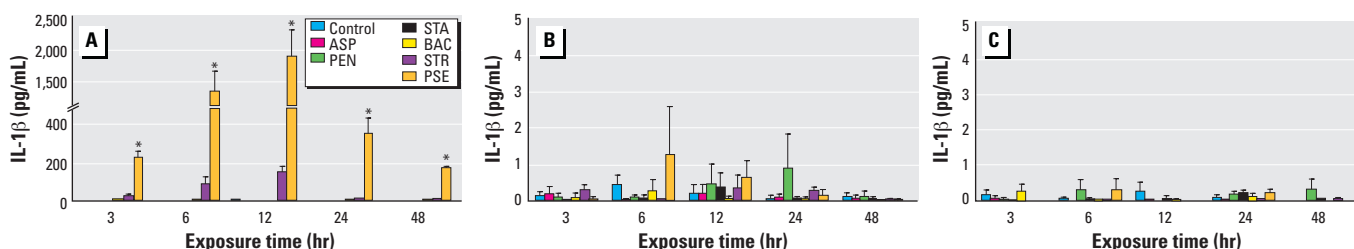


Figure 3. The time frame of IL-1 β production after exposure to a single dose (10^6 microbes/mL) of *A. versicolor* (ASP), *P. spinulosum* (PEN), *Sta. chartarum* (STA), *B. cereus* (BAC), *Str. californicus* (STR), and *Ps. fluorescens* (PSE) in (A) mouse RAW264.7 macrophages, (B) human 28SC cells, and (C) human A549 lung epithelial cells. Each column represents mean $\pm$ SEM of three independent experiments done in duplicate.

*Statistically significant difference from control, $p < 0.05$.

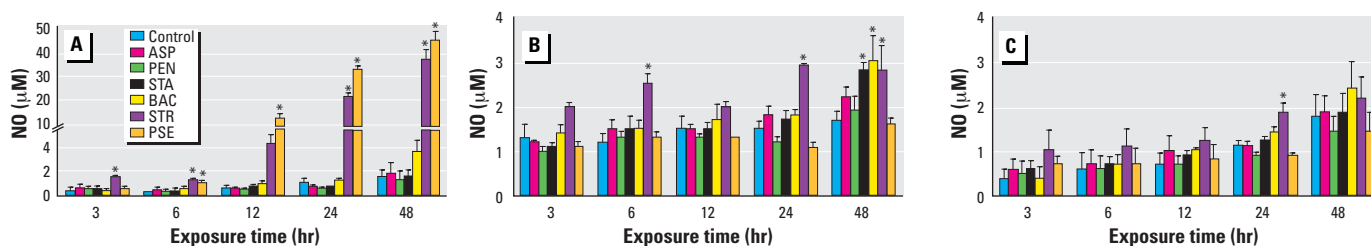


Figure 4. The time frame of nitrite production after exposure to a single dose (10^6 microbes/mL) of *A. versicolor* (ASP), *P. spinulosum* (PEN), *Sta. chartarum* (STA), *B. cereus* (BAC), *Str. californicus* (STR), and *Ps. fluorescens* (PSE) in (A) mouse RAW264.7 macrophages, (B) human 28SC cells, and (C) human A549 lung epithelial cells. Each column represents mean $\pm$ SEM of three independent experiments done in duplicate.

*Statistically significant difference from control, $p < 0.05$.

fungi used in this study lacked the capability to induce strong inflammatory responses.

The time- and dose-dependent production of cytokine IL-6 in all three cell lines was induced only by the spores of gram-positive bacterium *Str. californicus*. Obviously, this microbe possesses an exceptional immunostimulatory capacity. This observation is in line with our earlier findings demonstrating immunological activity of both live and dead spores from different strains of streptomycetes in mouse macrophages (Hirvonen et al.

1997) and in human A549 cells (Jussila et al. 1999). This capacity, however, is not common to all gram-positive bacteria because *B. cereus* did not activate either human or mouse cells to produce these mediators to the same extent as *Str. californicus*. The specific components or metabolites in the spores of streptomycetes causing these responses remain to be identified. For example, streptomycetes have complex lipid-sugar structures in their cell walls (Batrakov and Bergelson 1978), which bear a similarity to gram-negative bacterial LPS.

These bacteria also produce a vast variety of bioactive compounds as their secondary metabolites (Anderson and Wellington 2001). The spores of *Str. californicus* were also cytotoxic to mouse macrophages, but they decreased the viability of human cells only slightly.

In addition to the spores of gram-positive *Str. californicus*, the gram-negative bacteria *Ps. fluorescens* also caused strong inflammatory responses assessed as NO and cytokine production. These responses were induced most notably in mouse macrophages (IL-1, TNF, and IL-6 all increased). In human macrophages and epithelial cells only the production of IL-6 increased. It is most likely that the LPS is the active component in *Ps. fluorescens* because the profile and production of inflammatory mediators were almost identical.

The spores of gram-positive bacteria *B. cereus* proved to be the least potent of the bacterial strains, causing only slight production of cytokine and NO in mouse macrophages when compared to both *Ps. fluorescens* and *Str. californicus*. The inflammatory potential of the spores of *B. cereus* has not been previously studied, but this species is able to produce toxins (Andersson et al. 1999). In the current study, *B. cereus* was nearly as cytotoxic to mouse macrophages as *Str. californicus* and even more toxic in human epithelial cells at the highest tested doses. Both gram-positive and gram-negative bacterial lipoproteins in general have profound immunoregulatory functions, which are thought to be mediated by toll-like receptors (Brightbill et al. 1999).

In contrast to the bacterial strains, the spores of the fungal strains induced only a slight cytokine and NO production, and the cytokine profile was different. They were, however, cytotoxic, suggesting that the stimulation of cytokine production and cytotoxicity might not be associated. The cytotoxicity of fungi decreased in the order *Stachybotrys chartarum* > *Aspergillus versicolor* > *Penicillium spinulosum*, but it did not differ notably from the cytotoxicity of bacteria. The predominant cytotoxicity of the spores of *Sta. chartarum* over stimulation of the cytokine production in RAW264.7 macrophages has been observed previously (Ruotsalainen et al. 1998). Strain dependence of the toxicity of *Sta. chartarum* is well known (Gravesen et al. 1994; Jarvis et al. 1998; Nikulin et al. 1997; Ruotsalainen et al. 1998), but the strain dependence seems to apply also for these other fungi. The cytotoxicity of the strains of *Sta. chartarum*, *A. versicolor*, and *P. spinulosum* used in the present study varied when grown on different gypsum boards (Murtoniemi et al. 2001). The growth conditions of a microbe regulate its secondary metabolism, such as toxin production, and this regulation may be the key issue in understanding the microbial ecology

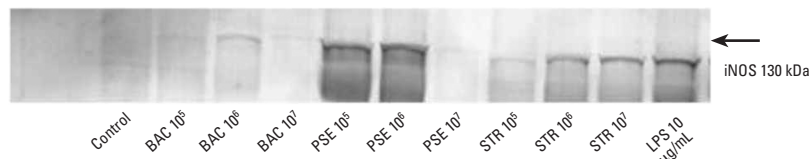


Figure 5. Dose response of iNOS expression in RAW264.7 macrophages after the 24-hr exposure to bacteria *B. cereus* (BAC), *Ps. fluorescens* (PSE), and *Str. californicus* (STR). LPS was used as a positive control. The figure is a representative example.

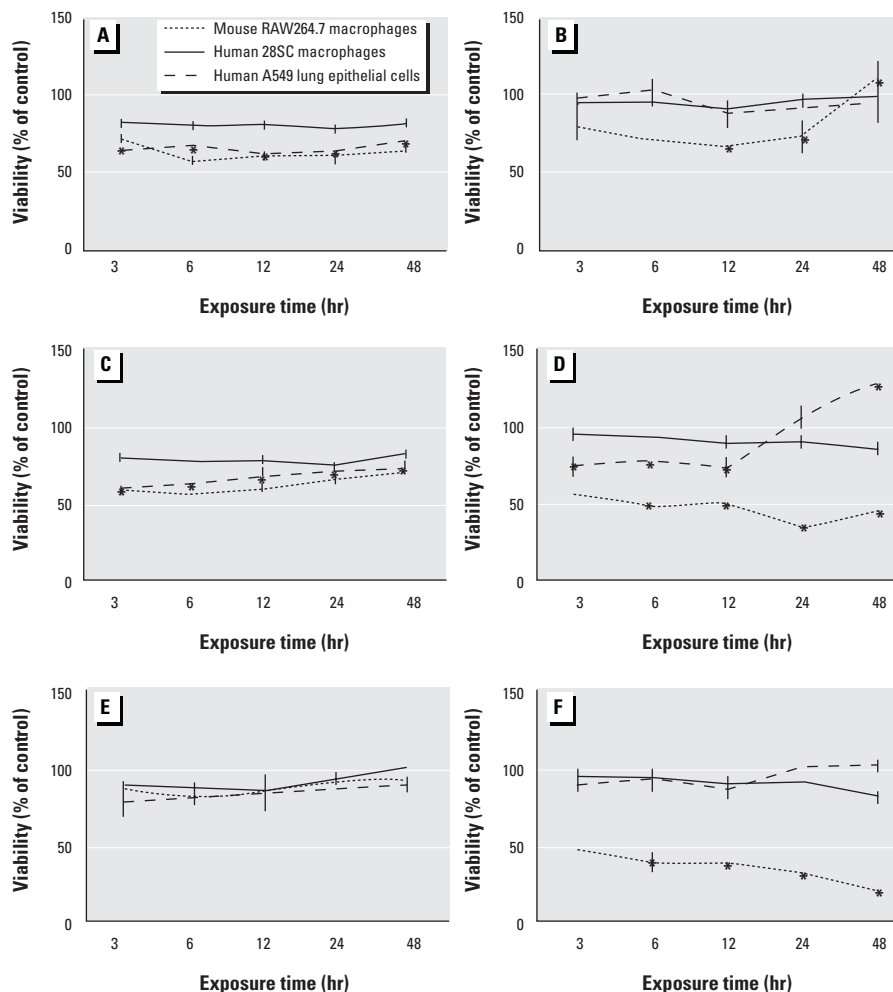


Figure 6. Decrease in cell viability induced by exposure to single doses (10^6 microbes/mL) of *A. versicolor*, *P. spinulosum*, *Sta. chartarum* (A,C,E), *B. cereus*, *Str. californicus*, and *Ps. fluorescens* (B,D,F) in mouse RAW264.7 macrophages, human 28SC macrophages and human A549 lung epithelial cells during a 48-hr period. Each point represents mean $\pm$ SEM of three independent experiments done in duplicate.

*Statistically significant difference from control, $p < 0.05$.

of moisture-damaged building materials (Hirvonen et al. 2001). Altered growth and sporulation environments for microbes may provoke normally innocuous microbes to produce components and/or metabolites that trigger inflammatory responses and cytotoxicity. This implies that the potential to cause adverse effects may be site specific.

There is some evidence pointing to a connection between exposure to 1 $\rightarrow$ 3- β -D-glucans, the cell wall component of fungi and some bacteria, and inflammatory-related health factors, including induction of cytokine production in blood monocytes and airway eosinophilia (Fogelmark et al. 2001; Rylander and Lin 2000). However, because most fungi and yeasts and some bacteria contain 1 $\rightarrow$ 3- β -D-glucans, it is obvious that this component cannot solely account for the significant differences within and between bacterial and fungal strains in their ability to trigger inflammatory responses in macrophages detected in this study. Other components of the bacterial cell wall (peptidoglycan) or fungal cells (ergosterol) have also been analyzed from mold exposure environments, but there is no known etiological connection to the symptoms, and thus these are only markers for the biomass (Fox and Rosario 1994; Pasanen et al. 1999).

The present results demonstrate clearly that mouse macrophages reacted more than human cells to exposure to different microbes. The production of IL-1 β and TNF- α was induced in mouse macrophages but not in human macrophages and epithelial cells. In addition, the response was much more intense in mouse macrophages. All these cells produced NO, but the iNOS synthesis was induced only in mouse macrophages, even by LPS. Among the measured inflammatory mediators, the production of the proinflammatory cytokine IL-6 was the most consistent indicator of inflammatory reactions. Its production was induced in all cell lines. The apparent insensitivity of human cells is most likely attributable to species differences in the regulation of NO and cytokine production. Species differences have been described in activation of lung macrophages to produce NO (Dörger et al. 1997; Jesch et al. 1997), and the regulation of the expression of iNOS is different in mouse and human cells (DeVera et al. 1996; Ganster et al. 2001; Lowenstein et al. 1993; Xie et al. 1993). Moreover, the maximal cytokine production requires several coincidental stimuli (Xie et al. 1993; Lowenstein et al. 1993; Gutierrez et al. 1995), and *in vitro* conditions cannot mimic such extracellular milieu as those found in the lungs. Although the human cell lines were primed with interferon- γ , this alone might not be sufficient; other priming agents would also be needed. Interferon- γ clearly potentiated the response

of *Str. californicus* in A549 lung epithelial cell line in our previous study (Jussila et al. 1999). Human cells were also more resistant to microbe-induced cytotoxicity than mouse macrophages. However, the setup of the experiments did not allow for the evaluation of the cellular mechanisms of these effects. The microbe-induced production of IL-6 in the human cell lines seems to be independent of the production of IL-1 β and TNF- α , although this needs to be confirmed by more sensitive methods.

In summary, both human and mouse macrophages responded to essentially the same stimulus, the bacterial exposure. On the basis of equivalent numbers, the studied bacterial strains were more potent than spores of fungi to induce production of proinflammatory mediators in cell cultures *in vitro*. The dose responses indicate that the potency decreased in the order *Ps. fluorescens* > *Str. californicus* > *B. cereus* > *Sta. chartarum* > *A. versicolor* > *P. spinulosum*. In the potency-to-cause cytotoxicity, no such clear systematic difference was observed, and the order was *Ps. fluorescens* > *Sta. chartarum* > *Str. californicus* > *A. versicolor* > *B. cereus* > *P. spinulosum*. Both the bacteria and fungi triggered the production of proinflammatory mediators at lower concentrations than needed for cytotoxicity, indicating that inflammation may be the primary response in lungs. These results imply that bacterial species need to be considered as causative agents for adverse inflammatory effects in water-damaged buildings.

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