Synergistic Interaction in Simultaneous Exposure to *Streptomyces californicus* and *Stachybotrys chartarum*

Kati Huttunen,[1,2] Jukka Pelkonen,[2,3] Kristian Fogg Nielsen,[4] Ulla Nuutinen,[2] Juha Jussila,[1] and Maija-Riitta Hirvonen[1]

[1]Department of Environmental Health, National Public Health Institute, Kuopio, Finland; [2]Department of Clinical Microbiology, University of Kuopio, Kuopio, Finland; [3]Department of Clinical Microbiology, Kuopio University Hospital, Kuopio, Finland; [4]Biocentrum-DTU, Technical University of Denmark, Lyngby, Denmark

The microbial exposure associated with health complaints in moldy houses consists of a heterogeneous group of components, including both living and dead bacteria, fungi, and their metabolites and active compounds. However, little is known about the interactions between different microbes and their metabolites, although the cytotoxicity and inflammatory potential of certain individual microbes have been reported. In this study, we investigated the inflammatory responses of mouse RAW264.7 macrophages after exposure to six indoor air microbes (*Aspergillus versicolor*, *Penicillium spinulosum*, *Stachybotrys chartarum*, *Bacillus cereus*, *Mycobacterium terrae*, and *Pseudomonas fluorescens*) alone and together with the actinomycete *Streptomyces californicus*. The production of nitric oxide, levels of the proinflammatory cytokines tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6), and cytotoxicity were measured. The coexposure to *Sta. chartarum* and *Str. californicus* caused a synergistic increase in the production of IL-6 but not other cytokines. In further experiments, the metabolites from *Sta. chartarum* or from closely related fungi (atranones B and E, satratoxin G, trichodermin, 7-α-hydroxytrichodermol, staplabin, and SMTP-7) and the known fungal toxins sterigmatocystin, citrinin, and ochratoxin A were each tested with *Str. californicus*. The testing revealed a synergistic response in TNF-α and IL-6 production after coexposure to *Str. californicus* with both trichodermin and 7-α-hydroxytrichodermol. Finally, the synergistic inflammatory response caused by *Str. californicus* and trichodermin together was studied by analyzing for the presence of nuclear factor-κB (NF-κB) in nuclear extracts of the exposed cells. The exposure to *Str. californicus* induced the binding of NF-κB proteins to the NF-κB consensus sequence as well as to the natural NF-κB site of the IL-6 promoter. Adding trichodermin to the exposure did not increase the DNA binding. *Key words:* bacteria, fungi, inflammation, interaction, mycotoxins, toxicity. *Environ Health Perspect* 112:659–665 (2004). doi:10.1289/ehp.6701 available via *http://dx.doi.org/* [Online 20 January 2004]

Microbial exposure in moldy houses consists of a heterogeneous group of compounds, including both living and dead bacteria, fungi, and their metabolites and active components as well as fungal microparticles (0.3–1 μm in diameter; Gorny et al. 2002; Kildesø et al. 2003). Interactions between the different exposures in a moisture-damaged house are inevitable, knowing that the spores of a single fungal species alone may contain various metabolites, and the moisture-damaged site is always a habitat of more than one microbial species (Andersson et al. 1997; Hyvärinen et al. 2002; Nielsen et al. 1999). Because many of the secondary metabolites are thought to be involved in chemical signaling between organisms or species, the production of some of these active metabolites may be enhanced or inhibited when the microbes interact (Christophersen 1996). However, little is known about the interactions between different elements of microbial exposure, although the inflammatory potential of some individual microbes and microbial components is acknowledged (Fogelmark et al. 2001; Jagielo et al. 1996).

The interactions between different microbial exposures have been studied previously by Norn (1993), who concluded that the exposure to fungal spores enhances the histamine release triggered by both allergic and nonimmunologic mechanisms in the cultured leukocytes of studied subjects. Also, there is evidence of a synergistic effect of gram-negative bacterial endotoxin and β-(1→3)-D-glucan on the influx of inflammatory cells into the alveolar space in the lungs of guinea pigs (Fogelmark et al. 1994, 2001). Furthermore, additive or synergistic effects of fungal metabolites have been suggested to be involved in the toxicity of contaminated feed materials (Foster et al. 1986) and in the effects of co-occurring fungal metabolites on insects (Dowd et al. 1989).

At the cellular level, microbes are recognized by the cells of the innate immune system by pattern-recognition receptors, which include signaling transmembrane proteins named Toll-like receptors (TLRs). Currently, there are 10 known human TLRs, which recognize and respond to different microbial products including microbial lipoproteins, peptidoglycan mainly from gram-positive bacteria, zymosan from yeast, and lipopolysaccharide from gram-negative bacteria (O'Neill 2002). Activated TLRs generate signals through several signal transduction pathways, including the proapoptotic caspase cascades and the Jun N-terminal kinase (JNK)/activator protein-1 (AP-1)– and nuclear factor-κB (NF-κB)–inducing pathways (Silverman and Maniatis 2001). The signaling pathways are similar to those generated by the proinflammatory cytokine IL-1, beginning with the recruitment of MyD88 to the Toll/IL-1 receptor domain and resulting in the degradation of inhibitory κB and translocation of NF-κB to the nucleus. In the nucleus, NF-κB binds to consensus sequences in the promoter regions of genes important for the inflammatory response, such as the cytokines tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) (O'Neill 2002).

Our earlier studies comparing the effects of fungal and bacterial exposure *in vitro* indicated that the importance of exposure to bacteria, especially gram-positive actinomycetes, might have been underestimated (Huttunen et al. 2003). Because the numbers of bacteria in indoor air are frequently higher than the number of fungi, exposures to bacteria and their interactions with fungi are of great importance (Flannigan et al. 1991). Actinomycetes species have sparked interest in many aspects of the moldy house problem. Their occurrence is considered to be indicative of moisture damage; several studies have consistently found actinomycetes in moldy house samples (Hyvärinen et al. 2002; Nevalainen et al. 1991), and very active metabolites have been isolated from actinomycetes-rich samples (Andersson et al. 1998). Furthermore, the inflammatory potential of these gram-positive sporulating bacteria has been studied in cell culture studies and in a mouse model, both studies revealing its high potential for inducing inflammatory effects (Huttunen et al. 2003;

Address correspondence to K. Huttunen, Division of Environmental Health, National Public Health Institute, P.O. Box 95, Neulaniementie 4, FIN-70701 Kuopio, Finland. Telephone: 358-17-201320. Fax: 358-17-201265. E-mail: Kati.Huttunen@ktl.fi

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Jussila et al. 1999, 2001). The frequent occurrence of actinomycetes in moisture-damaged materials means that they often share their habitat with other microbial species, especially fungal genera such as *Aspergillus*, *Penicillium*, and *Stachybotrys* (Hyvärinen et al. 2002).

Among the fungal species indicative of moisture damage, *Stachybotrys chartarum* has been a focus of interest in many toxicologic studies, mainly because of its ability to produce various mycotoxins including satratoxins, which belong to the group of macrocyclic trichothecenes known to have severe cytotoxic capabilities (Jarvis 2002). The macrocyclic trichothecenes are produced only by 30–40% of the *Sta. chartarum* strains isolated from buildings (Andersen et al. 2002, 2003). Trichothecenes have been suggested to be the cause of respiratory tract bleeding in studied cases of inhalation exposure to *Sta. chartarum* (Sorenson et al. 1987). Among the metabolites of *Stachybotrys* are various compounds with obscure biologic activities: the simple trichothecenes trichodermol and trichodermin, which are precursors to the satratoxins; the diterpenoid dollabellanes and atranones; and a very large number of spirocyclic drimanes including *Stachybotrys* lactones and lactams (Hinkley et al. 2000; Nielsen et al. 1999).

The aim of this study was to examine the interaction between indoor air microbes in mouse RAW264.7 macrophages during simultaneous exposure to the gram-positive bacteria *Streptomyces californicus* together with *Aspergillus versicolor*, *Penicillium spinulosum*, *Sta. chartarum*, *Bacillus cereus*, *Mycobacterium terrae*, or *Pseudomonas fluorescens*. Furthermore, atranones B and E, satratoxin G, and trichodermin (*Sta. chartarum* metabolites); 7-α-hydroxytrichodermol, staplabin, and SMTP-7 (from related species and genera, e.g., *Myrothecium roridum* and *Stachybotrys microspora*); and the known mycotoxins sterigmatocystin, citrinin, and ochratoxin A (metabolites of, e.g., *Aspergillus* spp. and *Penicillium* spp.) were all tested for their inflammatory and cytotoxic potency alone and together with *Str. californicus*. Finally, the synergistic inflammatory response caused by coincubation with *Str. californicus* and trichodermin was studied further by analyzing the presence of NF-κB in nuclear extracts of the exposed cells.

Materials and Methods

Microbial strains. The spores of three fungal strains, *Aspergillus versicolor* (HT486), *Penicillium spinulosum* (HT581), and *Sta. chartarum* (HT580 = IBT 9706, previously identified as a strain producing trichodermol and atranones but not macrocyclic trichothecenes), and spores/cells of bacterial strains *M. terrae* (BA26), *B. cereus* (B64), *Ps. fluorescens* (N78), and *Str. californicus* (A4) were all isolated and identified in previous studies (Huttunen et al. 2001, 2003). The fungal strains were cultured on 2% malt extract agar and bacterial strains on tryptone yeast glucose agar as a dense culture and incubated in the dark at 25°C for 7 days and 20°C for 5 days, respectively. All strains are available from the culture collection of the National Public Health Institute, Kuopio, Finland.

Fungal metabolites. Sterigmatocystin, citrinin, and ochratoxin A were obtained from Sigma (St. Louis, MO, USA). Trichodermin was a gift from Løvens Kemiske Fabrik A/S (Ballerup, Denmark); atranones B and E, 7-α-hydroxytrichodermol, and satratoxin G were a gift from B.B. Jarvis (University of Maryland, College Park, MD, USA); staplabin and SMTP-7 were a gift from K. Hasumi (Tokyo Noko University, Tokyo, Japan). Fungal metabolites were dissolved in methanol and diluted further in Hanks' balanced salt solution (HBSS), the final concentration of methanol being < 1%.

The samples from the microbial/fungal cultures (10^9 spores/mL) were extracted by ethyl acetate (analytical grade), evaporated to dryness, redissolved in methanol, and filtered through a 0.45 µL tetrafluoroethylene syringe filter (Titan 44513-PL; SRI, Eatontown, NJ, USA). Samples were then analyzed by reversed-phase chromatography on an Agilent 1100 liquid chromatographic (LC; Waldbronn, Germany) system with a diode array detector coupled to a Micromass LCT (Manchester, UK) orthogonal time-of-flight mass spectrometer operated in positive electrospray mode scanning from *m/z* 100 to 2,000. Peaks in the ultraviolet and mass spectroscopic traces were compared with reference standards (550 fungal metabolite reference standard from the Mycology Group metabolite database; Nielsen and Smedsgaard 2003).

Cell culture. The mouse macrophage cell line RAW264.7 was obtained from American Type Culture Collection (Rockville, MD, USA). The cells were cultured at 37°C in 5% carbon dioxide atmosphere in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2 mM), and penicillin–streptomycin [100 U/mL; all from Gibco, (Paisley, UK)]. The macrophages (5×10^5 cells/mL) were dispensed to six-well plates, 2 mL/well. The cells were allowed to adhere for 24 hr, and fresh complete medium was added before exposure. The cells were exposed to the combination of *Str. californicus* and graded doses of either microbes or fungal metabolites in 200 µL HBSS. After exposure, the adherent cells were resuspended in the culture medium by scraping, the viability of the cells was assessed, and the cell suspension was centrifuged (5 min, 8,000 rpm) to separate the cells from the culture medium. The samples were stored at –80°C for the subsequent analyses.

Study design. The macrophages were exposed to three doses (10^4, 10^5, and 10^6 spores or bacterial cells/mL) of each microbe alone and in combination with *Str. californicus* at a dose of 10^5 spores/mL for 24 hr. The interaction with the isolated fungal metabolites was tested by exposing the cells to three doses (10, 100, and 1,000 ng/mL) of each metabolite, except for satratoxin G, which was tested with doses 1, 10, and 100 ng/mL because of its toxicity. Trichodermin and its analog 7-α-hydroxytrichodermol were tested further also with additional doses (5, 50, and 500 ng/mL) to better define the dose response. The control cells were exposed to carrier buffer (10% methanol in HBSS), the final concentration of methanol in cell culture being 1%. The nuclear extracts for electrophoretic mobility shift assay (EMSA) were prepared from cells exposed to two doses (100 and 500 ng/mL) of trichodermin and 10^5 spores/mL *Str. californicus* for 24 hr both alone and together.

Nitrite analysis. Nitric oxide (NO) was measured 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 543 nm wavelength, which was measured with an enzyme-linked immunosorbent assay (ELISA) microplate reader (iEMS Reader MF; Labsystems, Turku, Finland). The production of nitrite was quantified by comparing the results with absorbances of standard solutions of sodium nitrite. Samples of at least three independent experiments were analyzed in duplicate.

Cytokine analysis. Proinflammatory cytokines TNF-α and IL-6 were analyzed from the cell culture medium immunochemically with commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) as described previously (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. Samples of at least three independent experiments were analyzed in duplicate.

Cell viability. The viability of the macrophages was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test to detect living mitochondria (Mosmann 1983). Functional mitochondria can transform MTT (Sigma) to formazan, which can be measured with a spectrophotometer. The proportion of viable cells in exposed samples was compared with control samples. To assure the quality of the controls, viability of the control samples

was measured by Trypan blue dye exclusion method where the viable (unstained) cells were counted in a hemocytometer under a light microscope. Samples of at least three independent experiments were analyzed in duplicate.

Preparation of nuclear extracts. For the preparation of nuclear extracts, the exposed cells were washed with Dulbecco's phosphate-buffered saline (pH 7.4; Gibco) and suspended in a hypotonic buffer [1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 10 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES)]. After a 5-min incubation on ice, 0.08% detergent NP-40 was added and the suspension was incubated for a further 2 min before centrifugation (400 × *g*, 2 min, 4°C). Pellets containing the nuclei were suspended in hypertonic buffer [25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM ethylenediamine tetraacetic acid (EDTA), 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, 420 mM NaCl, 20 mM HEPES] and incubated at 4°C for 30 min with gentle shaking. Finally, samples were centrifuged (25,000 × *g*, 20 min, 4°C), and supernatants were stored at –80°C. The amount of nuclear proteins was analyzed with Lowry's method (DC protein assay; BioRad Laboratories, Hercules, CA, USA).

EMSA assay. Double-stranded oligonucleotide containing the NF-κB consensus binding site was 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ (Promega, Madison, WI, USA). Single-stranded oligonucleotides containing the natural NF-κB binding motif of the mouse IL-6 promoter (sense 5′-AAA TGT GGG ATT TTC CCA TGA GTC-3′, antisense 5′-GAC TCA TGG GAA AAT CCC ACA TTT-3′) were annealed before labeling. The 173 bp region (–43 to –216) of the mouse IL-6 promoter was amplified by half-nested polymerase chain reaction (PCR) using BALB/c mouse genomic DNA (from J. Pelkonen, Kuopio, Finland) as the template. The primers were as follows: IL-6 sense, 5′-CGACGTCA-CATTGTGCAATC-3′; IL-6 antisense 1, 5′-CAGAATGAGCTACAGACATC-3′; IL-6 antisense 2, 5′-GTTGGGAGTGGTATC-CTCTG-3′.

The PCR product was extracted from low-melting agarose by the phenol–chloroform method. Double-stranded DNA oligonucleotides and the PCR product were labeled with [γ-^{32}P]ATP (adenosinetriphosphate, 3,000 Ci/mmol; Amersham Pharmacia Biotech, Roosendal, the Netherlands) using T4polynucleotide kinase (MBI Fermentas, Hanover, MD, USA). Labeled probes were separated using Probe Quant G-50 micro columns (Amersham Pharmacia Biotech) before their use in EMSA experiments.

Extracted nuclear proteins (6 μg protein per reaction) were incubated for 20 min with [γ-^{32}P]ATP–labeled probes in binding buffer (10% glycerol, 1 mM DTT, 1 mM EDTA, 25 mM HEPES, 100 mM NaCl) and 1.5 μg poly(dI-dC) in a final reaction volume of 20 μL.

The protein–DNA complexes were separated (25 mA, 90–120 min) on a high-ionic-strength gel (6% acrylamide) in running buffer (50 mM Tris, 380 mM glycine, 1 mM EDTA). After electrophoresis, the gel was dried (1 hr, 60°C) and exposed to autoradiography film for 1–2 days at –80°C.

In addition, for the supershift assay, the antibodies directed to the NF-κB family proteins p50 (SC-1190X), p65 (SC-109X), c-Rel (SC-6955X), Rel-B (SC-226X), and p52 (SC-7386X; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were preincubated with nuclear extracts for 10 min on ice before the addition of the radiolabeled probe.

Statistical analysis. The data were statistically analyzed using analysis of variance (ANOVA) and Tukey's test to compare the exposure to microbes or toxins with control samples. The trend in each dose response was tested with nonparametric Jonckheere-Terpstra test, which tests whether *k* independent samples defined by a grouping variable are from the same population. The trend was considered to be statistically significant at $p < 0.05$ (version 10.1.3; SPSS Inc., Chicago, IL, USA).

The results of the coexposure to the actinomycete and microbes/toxins were compared with the individual results of both the microbe/toxin and actinomycete exposures by using two-way ANOVA. Interaction term of actinomycetes and microbes/toxins was included in the model to assess the possible synergy.

Results

Simultaneous exposure to* Str. californicus *and selected microbes. Effects of a low dose of *Str. californicus* (10^5 spores/mL) alone and in combination with three different doses of *A. versicolor*, *P. spinulosum*, and *Sta. chartarum* spores and bacterial strains *B. cereus*, *M. terrae*, and *Ps. fluorescens* were studied to find out if the microbes were interacting during the exposure.

Cytokine production. Exposure to *Str. californicus* alone at the dose of 10^5 spores/mL caused strong TNF-α production and moderate IL-6 production. Of the six tested microbes, only *Ps. fluorescens* caused markedly increased IL-6 and TNF-α production ($p < 0.001$) without coexposure. Interestingly, simultaneous exposure to the low dose of *Str. californicus* (10^5 spores/mL) together with *Sta. chartarum* caused the IL-6 to be elevated up to 150 ± 170 pg/mL, although the synergistic interaction did not reach statistical significance ($p = 0.086$). This same effect was not seen with the other measured cytokine, TNF-α. The coexposure to the other tested microbes did not potentiate any of the measured cytokine responses to spores of *Str. californicus* (Table 1).

NO production. The dose of 10^5 spores/mL of *Str. californicus* on its own caused only minor production of NO. Of the single exposures, only *Ps. fluorescens* caused an intense production of NO ($p < 0.001$). None of the tested combinations caused a significant increase in NO production, but there was a slight addition of response ($p = 0.022$) with simultaneous exposure to *P. spinulosum* and *Str. californicus* spores (Table 1).

Cell viability. Single exposure to the spores of *Str. californicus* at the dose of 10^5 spores/mL caused only a moderate decrease in cell viability. Of the tested microbes, higher doses of *Sta. chartarum* ($p < 0.001$) and *Ps. fluorescens* ($p < 0.001$) were the most cytotoxic, followed by *A. versicolor* ($p < 0.001$), *B. cereus* ($p = 0.001$), *P. spinulosum* ($p < 0.001$), and *M. terrae*. An interaction was detected in the cytotoxicity to the cells in simultaneous exposure to *Sta. chartarum* ($p = 0.009$), *A. versicolor* ($p = 0.008$), *P. spinulosum* (0.023), or *Ps. fluorescens* ($p = 0.023$) together with *Str. californicus* spores, but the nature of the interaction was merely additive (Table 1).

Detected metabolites. The *A. versicolor* extract was dominated by sterigmatocystin, 5-methoxy sterigmatocystin, versicolorins, and other members of the sterigmatocystin biosynthetic pathway. The *P. spinulosum* extract contained very few and low quantities of metabolites, of which only 6-methylcitreoisocumarin could be identified. Two unknown metabolites with molecular weights of 265 and 348 Da were also detected, which suggested that this strain is rather a *Penicillium glabrum* than a *P. spinulosum*. The *Sta. chartarum* extract was dominated by seven spirocyclic drimanes. The atranones 3,4-epoxy-6-hydroxy-dolabella-7,12-diene-one; 6-hydroxy-dolaballa-3,7,12-trien-14-one; and atranone A were also present, whereas no trichothecenes (e.g., trichodermin, satratoxins) were detected. In addition, two unknown metabolites with presumed molecular weight of 531 Da were also detected. In the *Ps. fluorescens* extract, a cyclic peptide with a molecular weight of 1,126 was detected along with three minor analogs with molecular weights of 1,111, 1,153, and 1151 Da. Nothing was detected in the *B. cereus*, *M. terrae*, and the *Str. californicus* extracts.

Simultaneous exposure to* Str. californicus *and fungal metabolites. Combinations of different microbes with the spores of *Str. californicus* revealed that only spores of *Sta. chartarum* had a clear synergistic effect on the production of an inflammatory mediator in mouse macrophages. To identify the active component in the *Sta. chartarum* spore exposure, three metabolites known from previous research to be commonly produced by the strain were used in further exposure studies, and the effects were compared with those of selected microbial toxins.

Cytokine production. The tested metabolites of *Sta. chartarum* caused significantly different cytokine responses in RAW264.7 cells. Atranones B and E caused hardly any production of TNF-α or IL-6 in macrophages by any tested doses, whereas trichodermin induced a dose-dependent, significant increase in TNF-α production ($p < 0.001$) and slight but significant IL-6 production ($p < 0.001$) alone, and this response was markedly increased with coexposure to spores of *Str. californicus* ($p < 0.001$ for both TNF-α and IL-6; Figure 1). The closely related component, 7-α-hydroxytrichodermol, triggered similar effects on IL-6 and TNF-α production ($p < 0.001$ for both), although the dose–response curve differed from that obtained with trichodermin. The cytokine production induced by trichodermin tended to decline with the highest doses, possibly due to the cytotoxicity of the exposure, whereas the exposure to 7-α-hydroxytrichodermol increased the levels of produced cytokines at all tested doses (Figure 1). Satratoxin G was sufficiently toxic to significantly decrease the production of TNF-α ($p < 0.001$) when compared with control cells. Staplabin, SMTP-7, sterigmatocystin, citrinin, and ochratoxin did not induce any significant production of IL-6 in RAW264.7 macrophages, and only ochratoxin A induced a slight increase in TNF-α production. None of these mycotoxins added to the inflammatory effect of the spores of *Str. californicus*; in contrast, even a slight decreasing trend was detected in TNF-α production (data not shown).

NO production. Exposure to low dose of *Str. californicus* (10^5 spores/mL) elevated slightly, but nonsignificantly, the levels of nitrite in the culture medium of the macrophages. None of the tested microbial metabolites induced any significant production of NO alone, and only atranone E induced a slightly increased NO production together with the spores of *Str. californicus*. Interestingly, cells exposed to trichodermin alone produced somewhat higher amounts of NO than did cells exposed to both trichodermin and the actinomycete (data not shown).

Cell viability. The cytotoxicity of the metabolites of *Sta. chartarum* followed the potency to induce inflammatory effects. Atranones were not cytotoxic either alone or when combined with spores of *Str. californicus*. The doses > 50 ng/mL of trichodermin and the doses > 500 ng/mL of 7-α-hydroxytrichodermol were significantly cytotoxic compared with control ($p < 0.001$), and the responses were dose dependent ($p < 0.001$; Figure 2). Satratoxin G was highly cytotoxic to macrophages ($p < 0.001$); the cytotoxic effect of satratoxin G could be seen already at doses 10 times lower than those of the other tested toxins. Exposure to staplabin and SMTP-7 alone decreased slightly, but not dose dependently, the viability of the macrophages, whereas ochratoxin, citrinin, and sterigmatocystin did not cause excessive cell death alone or when combined with the spores of *Str. californicus* (Figure 2 B; dose–response data not shown).

Role of NF-κB in the microbial exposure and in the regulation of IL-6 production. The activation of NF-κB during the microbial exposure and the role of NF-κB in the regulation of IL-6 production were analyzed with two oligonucleotides containing either the consensus NF-κB binding site (cκB) or the natural NF-κB binding site of mouse IL-6 promoter (κBmIL-6). The role of other transcription factors and their potential interaction with NF-κB complexes were analyzed by using a PCR fragment from mouse IL-6 promoter (–43 to –216), which contained the same NF-κB binding site as the oligonucleotide κBmIL-6 as well as other potential transcription factor binding sites. Two doses of trichodermin (100 and 500 ng/mL) with and without *Str. californicus* were analyzed.

Binding of nuclear proteins to cκB oligonucleotide. *Str. californicus* exposure caused a major increase in DNA binding of all three nuclear proteins or protein complexes (named NF-κB A, B, and C) that were already present in untreated cells (Figure 3A, lane 12), whereas trichodermin exposure alone caused less intense induction of the complexes A and B (Figure 3A, lane 3). Trichodermin and *Str. californicus* together caused a NF-κB complex formation similar to *Str. californicus* alone, although complex A was attenuated with the dose of 100 ng/mL (Figure 3A, compare lanes 2 and 4).

Supershift assays. Five members of the NF-κB/Rel family of proteins have been found to be expressed in mammalian cells. These NF-κB/Rel subunits are p65/Rel-A, c-Rel, Rel-B, p105/NF-κB1 (which can be processed to p50), and p100/NF-κB2 (which can be processed to p52). These subunits usually exist as protein hetero- or homodimers. The NF-κB subunits were examined using Abs specific for p65, p50, p52, Rel-B, and c-Rel in supershift assays. The *Str. californicus* inducible complex A was supershifted with the antibody against c-Rel. Antibodies to p65, p50, p52, and Rel-B had no effect on the complexes (Figure 3B). Thus, other complexes remained unidentified.

Binding of nuclear proteins to κBmIL-6 oligonucleotide. Exposure to either *Str. californicus* or trichodermin (100 ng/mL) or to both

Table 1. Production of nitrite, TNF-α, IL-6, and cytotoxicity induced by exposure to graded doses of six different microbes alone and together with *Str. californicus* in mouse RAW264.7 macrophages.

		Nitrite (μM)		Toxicity (% of control)		TNF-α (ng/mL)		IL-6 (pg/mL)	
Exposure	Dose (μL)	Alone	With *Str.*	Alone	With *Str.*	Alone	With *Str.*	Alone	With *Str.*
Control	100	1.8 ± 0.5	2.4 ± 0.7*	0	28 ± 6*	0.04 ± 0.01	5 ± 2*	BD	11 ± 5*
A. versicolor	10^4	1.6 ± 0.4	3.5 ± 1.1	7 ± 4	20 ± 2	0.1 ± 0.05	7 ± 3	BD	8 ± 3
	10^5	1.7 ± 0.6	2.4 ± 0.6	18 ± 2*	29 ± 2	0.3 ± 0.2	6 ± 2	BD	9 ± 5
	10^6	1.9 ± 0.5	2.8 ± 0.7	45 ± 2*	51 ± 3	0.7 ± 0.3	8 ± 3	BD	27 ± 13
P. spinulosum	10^4	1.3 ± 0.2	2.2 ± 0.3	1 ± 3	23 ± 4	0.1 ± 0.01	5 ± 2	BD	7 ± 3
	10^5	1.8 ± 0.5	1.9 ± 0.2	10 ± 2	29 ± 1	0.1 ± 0.02	4 ± 2	2 ± 1	6 ± 2
	10^6	1.4 ± 0.3	3.2 ± 0.7	33 ± 1*	41 ± 1	0.2 ± 0.06	6 ± 3	BD	11 ± 5
Sta. chartarum	10^4	1.8 ± 0.4	3.9 ± 1.2	–7 ± 5	20 ± 5	0.3 ± 0.1	5 ± 2	3 ± 2	9 ± 0.3
	10^5	1.3 ± 0.2	2.8 ± 0.7	2 ± 4	30 ± 6	0.2 ± 0.1	8 ± 4	4 ± 2	150 ± 170
	10^6	1.7 ± 0.1	2.4 ± 0.1	82 ± 4*	80 ± 3	0.6 ± 0.3	7 ± 3	5 ± 3	560 ± 270
M. terrae	10^4	1.7 ± 0.3	3.2 ± 0.6	3 ± 4	25 ± 1	0.04 ± 0.01	7 ± 5	2 ± 2	12 ± 5
	10^5	1.5 ± 0.4	3.6 ± 1.1	2 ± 4	34 ± 3	0.11 ± 0.03	3 ± 1	3 ± 1	8 ± 4
	10^6	1.9 ± 0.4	3.1 ± 0.4	10 ± 16	39 ± 13	1.3 ± 0.9	6 ± 4	6 ± 4	21 ± 8
B. cereus	10^4	2.0 ± 0.2	3.0 ± 0.6	–1 ± 6	28 ± 1	0.4 ± 0.2	6 ± 3	BD	7 ± 4
	10^5	2.0 ± 0.6	2.1 ± 0.2	16 ± 16	45 ± 11	0.8 ± 0.3	7 ± 4	BD	18 ± 9
	10^6	1.7 ± 0.3	3.2 ± 0.5	38 ± 1*	54 ± 1	6 ± 2	12 ± 4	26 ± 3*	72 ± 25
Ps. fluorescens	10^4	8.1 ± 4.1	14.5 ± 6.9	1 ± 6	41 ± 6	8 ± 4	12 ± 5	320 ± 140	820 ± 520
	10^5	34.1 ± 5.2*	36.3 ± 4.6	58 ± 5*	74 ± 3	33 ± 10*	30 ± 5	2,390 ± 350*	2,170 ± 90
	10^6	36.5 ± 1.9*	36.3 ± 1.8	70 ± 3*	83 ± 1	36 ± 6*	35 ± 8	2,090 ± 150*	2,160 ± 30

BD, below detection limit. Each column represents mean ± SE of three independent experiments.
*Statistically significant increase compared with control ($p < 0.05$).

stimuli simultaneously increased the DNA binding activity of complexes NF-κB A/B and NF-κB C (Figure 3C, lanes 1–4). Exposure to higher trichodermin concentration inhibited *Str. californicus*–induced DNA binding of complexes NF-κB A/B and NF-κB C (Figure 3C; compare lanes 2 and 6).

Binding of nuclear proteins to PCR fragment of IL-6 gene promoter. When testing the PCR fragment of IL-6 gene promoter, binding was altogether weak, and we presume that larger complexes were formed than with the short NF-κB oligonucleotides. However, trichodermin exposure caused more intense DNA binding activity of the complex NF-IL-6 B (Figure 3D).

Discussion

The present study was designed to investigate the effect of simultaneous exposure to microbes typically present in indoor air of moldy houses. The results show that, of the six studied microbial strains, only *Sta. chartarum* is able to potentiate the inflammatory effect of gram-positive bacterium *Str. californicus*. Furthermore, among the metabolites typically produced by *Sta. chartarum*, there is at least one compound, trichodermin, that produces a similar synergistic effect along with a closely related compound, 7-α-hydroxytrichodermol. The exposure to *Str. californicus* induces the nuclear binding activity of a well-known transcription factor NF-κB, including one complex with c-Rel and some unidentified subunits. Adding trichodermin to the exposure did not increase the DNA binding, hence leaving the mechanism behind synergistic effect of trichodermin and *Str. californicus* unclarified.

Interestingly, although spores of *Sta. chartarum* alone were not able to evoke any inflammatory responses in macrophages, they triggered a significant, dose-dependent cytokine response in these cells when exposed in conjunction with a low dose of *Str. californicus*. This is of special interest because it has been shown that the actinomycetes are found together with *Stachybotrys* in 60% of paper materials, indicating that the simultaneous exposure to these microbes in moldy houses is a likely scenario (Hyvärinen et al. 2002). However, coexposure to fungal and bacterial strains does not invariably evoke inflammatory responses in macrophages because the observed synergistic effect with *Str. californicus* was not seen with the other tested fungal strains, although both *Aspergillus* and *Penicillium* are also among the fungal strains occurring frequently with actinomycetes in moisture- and mold-damaged building materials (Hyvärinen et al. 2002). To study this phenomenon further, we tested the metabolites typically produced by *Sta. chartarum* to identify the active component(s) of these fungal spores responsible for the detected synergistic effects with gram-positive bacteria *Str. californicus*. Other fungal metabolites were analyzed for reference purposes, most of them being compounds produced by common indoor air fungal species.

Our results show that trichodermin, a metabolite of most *Sta. chartarum* strains (Andersen et al. 2002, 2003), mimics the dose-dependent synergistic inflammatory effects in macrophages produced by exposure to the whole spores of this fungus. The amplified cytokine responses were triggered in macrophages also by the closely related 7-α-hydroxytrichodermol. Exposure to the actinomycete *Str. californicus* increased the nuclear localization of NF-κB proteins, but the synergistic interaction between trichodermin and actinomycete could not be explained by the amount of NF-κB in the nucleus. Instead, trichodermin may act through a parallel signal transduction pathway leading to the binding of other transcription factors to IL-6 promoter and therefore enhancing transcription of the IL-6 gene.

These findings are in line with earlier interpretations suggesting that inhalation of fungi, particularly those that produce mycotoxins, may cause immunologic dysregulation in the intact animal or human. It has been suggested that exposure to mycotoxins may interfere with the function of immunologic cells, because a number of mycotoxins have been shown to activate macrophages *in vitro* (Ji et al. 1998; Rotter and Oh 1996; Wong et al. 1998). Moreover, in animal models a low-level exposure to the trichothecenes ochratoxin and sterigmatocystin has been linked to immune dysfunction and compromised host resistance (Pestka and Bondy 1990).

In the present study, the macrocyclic trichothecene satratoxin G was highly cytotoxic to exposed cells with and without bacterial exposure. Although the toxicity and inflammatory effect of satratoxin G exposure were not markedly changed by the presence of the actinomycete, the toxicity as such is noteworthy because the spores or other particles can act as a carrier of these toxins. Atranones B and E, immunosuppressive spirocyclic drimanes staplabin and SMTP-7, nephrotoxic citrinin, and carcinogenic sterigmatocystin did not induce

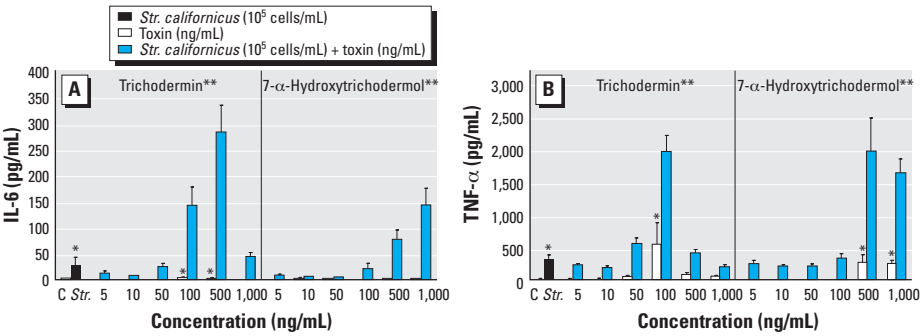

Figure 1. The production of IL-6 (*A*) and TNF-α (*B*) after exposure to six doses (5–1,000 ng/mL) of trichodermin and 7-α-hydroxytrichodermol alone and together with *Str. californicus* (10^5 spores/mL). Abbreviations: C, buffer control; *Str.*, 10^5 spores/mL *Str. californicus* alone. Each bar represents mean ± SE of five independent experiments done in duplicate.
*Statistically significant difference from control. **Statistically significant synergistic effect in simultaneous exposure ($p < 0.05$).

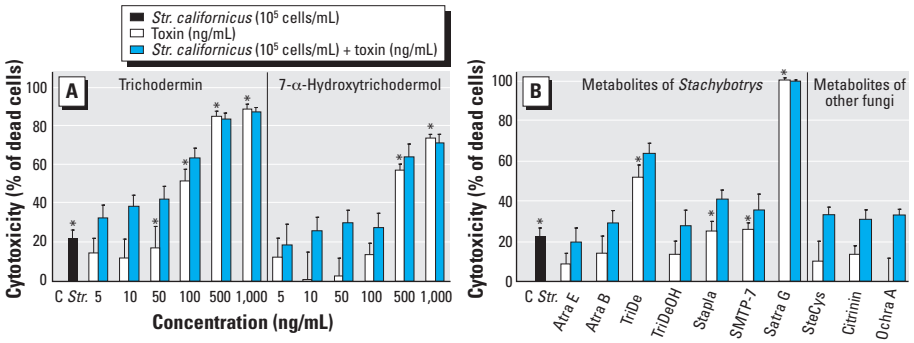

Figure 2. (*A*) The cytotoxicity of the exposure to six doses (5–1,000 ng/mL) of trichodermin and 7-α-hydroxytrichodermol alone and together with *Str. californicus* (10^5 spores/mL). (*B*) The cytotoxicity of the exposure to one dose (100 ng/mL) of each microbial metabolite alone and together with *Str. californicus* (10^5 spores/mL). Each column represents the mean ± SE of five independent experiments done in duplicate. Abbreviations: Atra B, atranone B; Atra E, atranone E; C, buffer control; Ochra A, ochratoxin A; Satra G, satratoxin G; Stapla, staplabin; SteCys, sterigmatocystin; *Str.*, 10^5 spores/mL of *Str. californicus* alone; TriDe, trichodermin; TriDeOH, hydroxytrichodermol.
*Statistically significant difference from control ($p < 0.05$).

any significant production of IL-6 or TNF-α in mouse macrophages, and the hepatotoxic agent ochratoxin A caused only a slight increase in TNF-α production in this study. None of these toxins supplemented the inflammatory effect of the spores of *Str. californicus*; on the contrary, the TNF-α production was, if anything, inhibited slightly.

Apart from the toxicity of the trichothecenes produced by *Stachybotrys*, their ability to act as inhibitors of protein synthesis has also been reported (Bamburg 1976; Wei and McLaughlin 1974). Interestingly, *Fusarium* mycotoxins, including also several trichothecenes, have been shown to inhibit the protein synthesis in mouse fibroblasts in a synergistic manner (Groten et al. 1998). Combining of trichothecene deoxynivalenon (DON) with other *Fusarium* metabolites has also been found to increase toxicity of the exposure to caterpillars, presumably via inhibition of their detoxifying enzymes (Dowd et al. 1989). Similar synergism has been observed with mycotoxin-induced porcine nephropathy, where penicillic acid inhibits the enzymes responsible for detoxification of ochratoxin A (Stoev et al. 2001). Furthermore, the exposure to the trichothecene DON has been studied previously *in vitro* with RAW264.7 mouse macrophages. The conclusion of that study was that the concurrent exposure to DON and bacterial lipopolysaccharide or interferon γ superinduced the production of TNF-α and IL-6 (Ji et al. 1998), which supports our present results concerning trichodermin.

A previous chemical study of metabolites from > 50 *Stachybotrys* isolates revealed that all produced high quantities of immunosuppressant spirodrimanes, but only one-third of the isolates produced the macrocyclic trichothecenes. Further studies on the non-macrocyclic-trichothecene–producing isolates led to the isolation of the novel diterpenoids called atranones (Andersen et al. 2002; Hinkley et al. 2000). The inflammatory responses to atranones have been studied previously in a chemical study of 20 *Stachybotrys* strains and the metabolites they produced. However, the inflammatory effect of nonsatratoxin–producing strains could not be attributed exclusively to the atranones (Nielsen et al. 2001). In the present study, trichodermin was not detected by LC–mass spectrometry in the sample of *Sta. chartarum*, but the observed effects of exposure to *Sta. chartarum* spores could be due to the accumulation of other simple trichothecenes from this very complex metabolic pathway. This question will remain unresolved until isolation of the biologically active metabolites has been performed.

Conclusions

Microbes are capable of producing various compounds with biologic activity, many of which are yet to be isolated and characterized. Both inactivation and stimulation of the pulmonary defense system are relevant for the overall physiologic consequences. For instance, extracts of fungal strains are known to have ciliostatic effects in tracheal organ cultures (Piecková and Jesenska 1998), indicating that the destruction of the defense mechanisms in the lungs could be one factor contributing to the symptoms experienced by occupants of moldy houses. The exposure route, typically inhalation in a moldy house, has also a significant role in the magnitude of the responses. In experimental studies, exposure via inhalation caused much more severe symptoms in animals when compared with exposure via systemic administration (Creasia et al. 1987, 1990).

One of the key elements of our *in vitro* studies has been the development of a relevant model for screening agents that may contribute to health-relevant exposure in moldy houses, including the interaction between the various possible components of microbial exposure. This study demonstrates that synergistic interaction between different exposure agents is a biologically plausible theory that could explain adverse health effects experienced at fairly low indoor airborne microbial concentrations. However, additional research is needed to further characterize such interactions in intact animals, as well as in human cell culture systems.

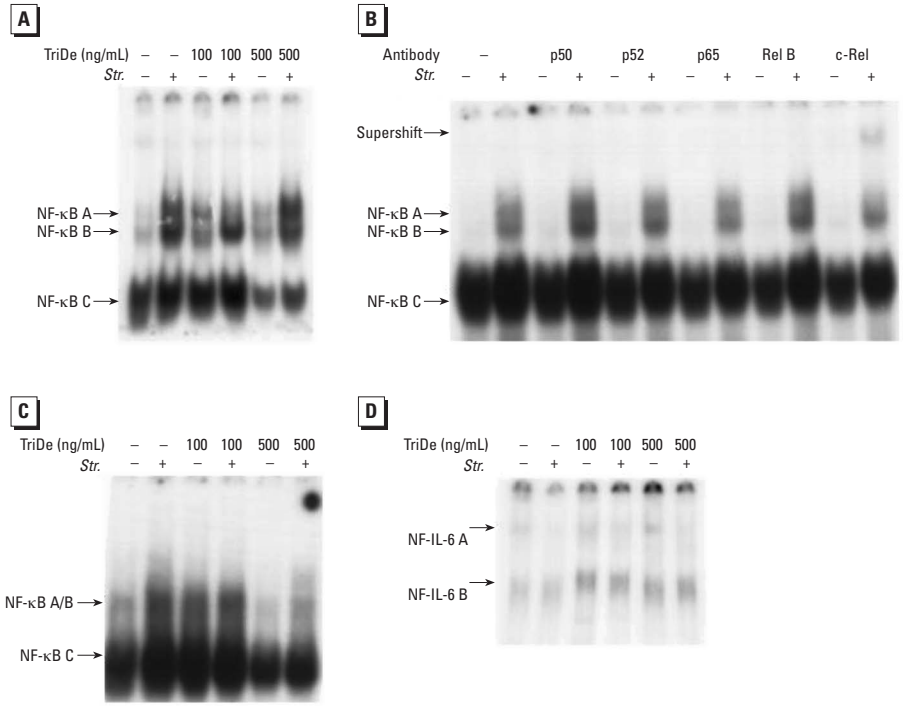

Figure 3. The nuclear binding activity of transcription factor NF-κB in cells exposed to *Str. californicus* alone and together with two doses of trichodermin. Abbreviations: *Str.*, 10^5 spores/mL of *Str. californicus*; TriDe, trichodermin. (*A*) Binding of nuclear proteins to consensus NF-κB oligonucleotide. (*B*) Supershift of NF-κB complexes by antibodies against p50, p52, p65, Rel B, and c-Rel. (*C*) Binding of nuclear proteins to natural NF-κB binding site of mouse IL-6 promoter. (*D*) Binding of nuclear proteins to PCR fragment of IL-6 gene promoter.

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