Inflammatory Responses in Mice after Intratracheal Instillation of Microbes Isolated from Moldy Buildings

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Academic dissertation

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ABSTRACT

Moisture-damage is common in buildings, and it is associated with a variety of adverse health effects, especially inflammatory responses in the lower airways. Exposure to microbial spores or cells has been suspected to be one reason for the inflammation, but the inflammatory and toxic potential of the microbes has not been well characterized.

In this study, a mouse model was devised to elucidate and compare the adverse effects provoked by microbes isolated from the indoor air of a moisture-damaged building. The animals were exposed intratracheally to the microbial spores or cells. The effects caused by the bacterial species *Streptomyces californicus* and *Mycobacterium terrae* and the fungi *Aspergillus versicolor* and *Penicillium spinulosum* were investigated. They are typical microbes observed in moisture-damaged buildings, but *P. spinulosum* is also frequently observed in all indoor air environments. Both the dose-response and time-course of the inflammatory and toxic responses were investigated after a single dose of the microbe. Biochemical parameters indicating inflammation and/or toxicity (tumor necrosis factor α, TNFα, interleukin-6, IL-6, total protein, albumin, hemoglobin and lactate dehydrogenase) were measured from bronchoalveolar lavage fluid (BALF), and a histopathological analysis was performed from lungs, lymph nodes and spleen. In addition, inducible nitric oxide synthase (iNOS) was determined from lavaged cells, and the cytokine concentrations were measured in serum. Moreover, the effects of *S. californicus* were studied after repeated dosing of the spores. In that experiment also lymphocyte subpopulations were investigated in the lungs, lymph nodes and the spleen.

Both the biochemical parameters and histopathology revealed that all the microbes studied provoked inflammation after a single dose, but the magnitude and its characteristic features were different. The spores of *S. californicus* provoked a very intense acute inflammation indicated by a strong and rapid IL-6 and TNFα production in the lungs, recruitment of inflammatory cells into the airways, and expression of inducible nitric oxide synthase (iNOS) in lavaged cells at 24 hours. An increase in TNFα and IL-6 concentrations in serum was also detected. The cells of *M. terrae* induced a biphasic inflammatory response, which consisted of an acute and a sustained phase. In the acute phase, TNFα and IL-6 were produced and inflammatory cells were recruited into the lungs. In the later phase, TNFα production was sustained up to 14 days, and inflammatory cell recruitment was even more intense, with iNOS being expressed in lavaged cells. Reactive changes in lymph nodes were also observed. The inflammatory response lasted until the end of the experiment (28 days), and at that time some mycobacterial cells were still present in the lungs.

At comparable volumetric doses with the bacteria, the fungal species also induced a rapid production of proinflammatory cytokines, and inflammatory cell recruitment into the airways, but they were generally less potent than the bacteria. *P. spinulosum* induced only a mild inflammatory response and transient TNFα and IL-6 production into BALF. The spores of *A. versicolor* caused a slow TNFα response, and the inflammatory cell response was more

sustained than by *P. spinulosum*. In contrast to the bacteria, the studied fungal species did not induce expression of iNOS.

Acute cytotoxicity in lungs indicated by LDH response was observed during *S. californicus*, *M. terrae* and *A. versicolor* exposure. *M. terrae* exposure caused the strongest and most sustained effect. However, none of the microbes were highly cytotoxic in the lungs, and the effect was frequently associated with an acute inflammatory response. *P. spinulosum* exposure showed no cytotoxic effect.

After repeated airway exposure to the spores of *S. californicus*, both the innate and adaptive host defenses were activated in the lungs. The inflammatory cell response in the lungs was more severe and appeared at a lower dose level than after a single dose. Spleen cell count was decreased indicating a systemic immunotoxic effect, and the lymphocyte subpopulations were altered in the lungs, lymph nodes and the spleen. Reactive changes were observed in lymph node histopathology.

In summary, the results show that the indicated microbes have a different potential to cause inflammatory and toxic responses after airway exposure in mice, and suggest that microbes present in a moisture-damaged building can induce inflammation in lungs and cause systemic toxicity.

To my family

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ABBREVIATIONS

A. versicolor	*Aspergillus versicolor*
ALL	alveolar lining layer
AP	alkaline phosphatase
a_w	water activity
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
BCIP	bromo chloro indolyl phosphate disodium
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CD	clusters of differentiation
cGMP	cyclic guanosine monophosphate
CO_2	carbon dioxide
DNA	deoxyribonucleic acid
EDTA	ethylene diaminetetra-acetic acid
ELISA	enzyme linked immuno sorbent assay
FBS	fetal bovine serum
FACS	fluorescence activated cell sorter
FITC	fluorescein isothiocyanate
g	acceleration of gravity
HBSS	Hank's balanced salt solution
HRP	horse radish peroxidase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
kDa	kiloDalton
LAM	lipoarabinomannan
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
M. terrae	*Mycobacterium terrae*
MHC	major histocompatibility complex
MVOC	microbial volatile organic compound
NBT	nitro blue tetrazolium
NK cell	natural killer cell
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
NOS	nitric oxide synthase
$OONO^-$	peroxynitrite
P. spinulosum	*Penicillium spinulosum*
PAF	platelet activating factor
PBS	phosphate buffered saline
PDE	phosphodiesterase
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PMSF	phenyl methyl sulfonyl fluoride
RAW264.7	mouse macrophage cell line
ROS	reactive oxygen species

rpm	revolutions per minute
S. californicus	*Streptomyces californicus*
SCG	single cell gel
SE	standard error
SDS	sodium dodecyl sulfate
SPF	specific pathogen free
spp.	species
TCR	T cell receptor
Th cell	helper T cell ($CD3^+CD4^+$)
Tk cell	killer T cell ($CD3^+CD8^+$)
TMB	tetramethylbenzidine
TNFα	tumor necrosis factor alpha
TXA_2	thromboxane A_2

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, referred to in the text by the Roman numerals I - V.

I Jussila, J., Komulainen, H., Huttunen, K., Roponen, M., Hälinen, A., Hyvärinen, A., Kosma, V.-M., Pelkonen, J., and Hirvonen, M.-R. Inflammatory responses in mice after intratracheal instillation of spores of *Streptomyces californicus* isolated from indoor air of a moldy building. Toxicol. Appl. Pharmacol. 2001;171:61-69.

II Jussila, J., Komulainen, H., Huttunen, K., Roponen, M., Iivanainen, E., Torkko, P., Kosma, V.-M., Pelkonen, J., and Hirvonen, M.-R. *Mycobacterium terrae* isolated from indoor air of a moisture-damaged building induces sustained biphasic inflammatory response in mouse lungs. Environ. Health Perspect. 2002; 110:1119-1125.

III Jussila, J., Komulainen, Kosma, V.-M., Nevalainen, A., Pelkonen, J., and Hirvonen, M.-R. Spores of *Aspergillus versicolor* isolated from indoor air of a moisture-damaged building provoke acute inflammation in mouse lungs. Inhal. Toxicol. 2002; 14:1261-1277.

IV Jussila, J., Komulainen, H., Kosma, V.-M., Pelkonen, J., and Hirvonen, M.-R. Inflammatory potential of the spores of *Penicillium spinulosum* isolated from indoor air of a moisture-damaged building in mouse lungs. Environ. Toxicol. Pharmacol. 2002; 12:137-145.

V Jussila, J., Pelkonen, J., Kosma, V.-M., Mäki-Paakkanen, J., Komulainen, H., and Hirvonen, M.-R. Systemic immunoresponses in mice after repeated lung exposure to spores of *Streptomyces californicus*. Clin. Diagn. Lab. Immunol. In press.

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ORIGINAL PUBLICATIONS

1 INTRODUCTION

Moisture-damage in buildings is common in different climates (e.g. Hunter et al., 1988; Dales et al., 1991a; Nevalainen et al., 1998). In Finland, signs of current or previous moisture-damage have been reported in up to 80% of houses (Nevalainen et al., 1998). Several epidemiological studies have indicated that there is an association between the adverse health effects experienced by the occupants and the presence of moisture-damage in buildings. In particular symptoms and diseases in the upper or lower airways have been observed, but a number of general symptoms have also been reported.

Excess moisture encourages microbial growth on building materials. Increased numbers of microbes or their spores, and atypical microbial species have been frequently detected in the indoor air of moisture-damaged buildings (e.g. Waegemaekers et al., 1989; Nevalainen et al., 1991; Hyvärinen et al., 1993; Samson et al., 1994). It is possible that the microbes have a role in inducing the adverse health effects observed among the occupants of such buildings. However, there is little toxicological data concerning these microbes. In particular, more *in vivo* data in experimental conditions during airway exposure are needed. The data from animal studies permit the analysis of whole lung and organism responses to the microbial exposures. In addition, the function of the complex immune system, composed of a network of diverse cell types, can be more thoroughly investigated. In this thesis *in vivo* effects induced by four microbes isolated from moisture-damaged buildings were evaluated to obtain new information for the health risk assessment.

2 REVIEW OF LITERATURE

2.1 Exposure in moisture-damaged buildings

The occupants of moisture-damaged buildings are exposed to different substances of microbial or other origin. Several microbes (e.g. fungi and actinobacteria) produce spores that can spread into indoor air. The occupants can also be exposed to microbial cells, hyphae or their fragments, since particles other than spores can be released from fungal cultures (Kildesø et al., 2000; Gorny et al., 2002). Microbes can produce toxic secondary metabolites (e.g. mycotoxins) and they possess bioactive surface structures (e.g. bacterial endotoxins) (Young et al., 1998; Peraica et al., 1999). Fungi and actinobacteria (previously known as actinomycetes) can also emit volatile organic compounds (MVOC's) (Pieckova and Jesenska, 1999; Scholler et al., 2002). In addition to microbial exposure, increased moisture and microbes may decompose building structures, and cause volatile emissions from the materials (Pasanen et al., 1998; Wolkoff and Nielsen, 2001). Exposure to mite allergens may also explain some of the adverse effects (Bornehag et al., 2001). Altogether, the relative importance of different indoor air exposures in provoking adverse health effects is not known. It has been especially difficult to obtain quantitative relationships between exposure and the symptoms. Moreover, several other factors, such as insufficient ventilation, high temperature and draft can influence the perception of indoor air quality and modify the effects caused by biological and chemical contaminants in indoor air of moisture-damaged buildings (Husman, 1996).

Dampness and visible mold growth in the buildings are frequently associated with increased levels of fungal spores in indoor air (Hunter et al., 1988; Hyvärinen et al., 1993; Li and Kendrick, 1995; Garrett et al., 1998; Dharmage et al., 1999; Hyvärinen et al., 2001a). Especially, during repair of moisture-damage, the number of microbes is markedly increased in indoor air (Rautiala et al., 1996). Both fungi (e.g. *Stachybotrys*, *Penicillium* and *Aspergillus*) and bacteria (e.g. actinobacteria (e.g. *Streptomyces*), mycobacteria and *Pseudomonas*) have been isolated from the indoor air and building materials of moisture-damaged buildings (Hunter et al., 1988; Nevalainen et al., 1991; Hyvärinen et al., 1993; Samson et al., 1994; Andersson et al., 1997; Hyvärinen et al., 2002; Rautiala et al., submitted). Even though the measured microbial concentrations are not necessarily high in the indoor air, the atypical, potentially toxigenic, microbial species may cause adverse health

effects even at lower concentrations (Husman, 1996). The microbial species, which are regarded as moisture-indicator microbes in the literature are listed in Table 1. Their appearance is partly dependent on the water activity (a_w) of a building material.

Table 1. Microbes regarded as moisture-indicator organisms (Modified from Samson et al., 1994).

- **Materials with a high water activity (a_w > 0.90 - 0.95)**
  Aspergillus fumigatus
  Actinobacteria
  Exophiala
  Fusarium
  Gram-negative bacteria (e.g. *Pseudomonas*)
  Phialophora
  Stachybotrys
  Trichoderma
  Ulocladium
  yeasts (*Rhodotorula*)

- **Materials with a moderate water activity (0.85 < a_w < 0.90)**
  Aspergillus versicolor

- **Materials with a lower water activity (a_w ≤ 0.85)**
  Aspergillus versicolor
  Eurotium
  Penicillia (e.g. *Penicillium chrysogenum*, *P. aurantiogriseum*)
  Wallemia

Several species of these microbes can produce toxic compounds. One of the most thoroughly investigated toxigenic fungus in indoor air is *Stachybotrys chartarum*, which can produce a diverse spectrum of toxins (Jarvis et al., 1995; Fung et al., 1998). *Aspergillus versicolor* and *Fusarium* species are other examples of toxin producing fungi (Samson et al., 1994). Moreover, Gram-positive bacteria streptomycetes have the capability to produce several different agents, including toxic substances (Arcamone, 1998; Paananen et al., 2000; Bolzan and Bianchi, 2001; Watve et al., 2001).

2.2 Adverse health effects associated with moisture-damage in buildings

The association between adverse health effects and exposure in moisture-damaged buildings has been shown in several epidemiological studies (Table 2). The epidemiological evidence is strongest for respiratory adverse effects (cough, wheeze and asthma) (Bornehag et al., 2001).

Moreover, a number of general symptoms have been reported. However, all adverse effects have apparently not been identified yet.

Table 2. Symptoms and diseases associated with moisture-damages in buildings.

Symptom / disease	Reference
• **Respiratory tract symptoms and diseases**	
- cough	(Brunekreef, 1992; Koskinen et al., 1999a, 1999b)
- wheeze	(Platt et al., 1989)
- asthma	(Waegemaekers et al., 1989)
- phlegm	(Brunekreef, 1992; Pirhonen et al., 1996)
- dyspnoea	(Platt et al., 1989)
- bronchitis	(Dales et al., 1991b)
- increased respiratory infections	(Koskinen et al., 1995, 1997)
- pulmonary hemosiderosis	(Etzel et al., 1998; Dearborn et al., 1999)
- hypersensitivity pneumonitis	(Park et al., 1994; Lee et al., 2000)
- hoarseness	(Koskinen et al., 1995, 1999a; Pirhonen et al., 1996)
- sore throat	(Platt et al., 1989)
- nasal congestion	(Platt et al,. 1989)
- nasal discharge	(Platt et al,. 1989)
- rhinitis	(Pirhonen et al., 1996)
• **Other symptoms and diseases**	
- skin symptoms	(Johanning et al., 1996)
- eye irritation	(Hodgson et al., 1998; Johanning et al., 1996)
- difficulties in concentrating	(Koskinen et al., 1999b)
- fatigue	(Koskinen et al., 1999b, Johanning et al., 1996)
- lethargy	(Hodgson et al., 1998)
- nausea	(Koskinen et al., 1999b)
- lumbar backache	(Pirhonen et al., 1996)
- recurrent stomachache	(Pirhonen et al., 1996)
- aching joints	(Platt et al., 1989)
- rheumatic diseases	(Roponen et al., 2001)

2.3 Potential mechanisms and other effects

The mechanisms behind most of the observed adverse health effects associated with exposure in moisture-damaged buildings remain unknown, with both immunological and non-immunological mechanisms being suspected (Damgård Nielsen et al., 1995).

Immunostimulation and allergy

Exposure to microbes and their toxic substances may trigger innate immune responses i.e. increased production of inflammatory mediators, such as cytokines, and reactive oxygen and nitrogen radicals in mammalian cells *in vitro* (Ruotsalainen et al., 1995; Hirvonen et al., 1997a, 1997b, 1997c; Huttunen et al., in press). These responses are important elements in the normal host defense, but sustained or excess release of inflammatory mediators and toxic radicals may provoke adverse health effects. For example, increased phagocytic clearance by inflammatory cells recruited to the site of exposure is an important component of host defense, but inflammatory cells can provoke immunopathological effects also to the host via their products (Stockley, 1995).

One of the best known immunological mechanism for an adverse health effect caused by fungal exposure is immunoglobulin E (IgE) -mediated immediate allergy (Gell and Coombs classification Type I). The most prevalent indoor air fungal species causing IgE-mediated allergy include *Aspergillus*, *Cladosporium*, and *Penicillium* (Ledford, 1994), though many other fungi have also been associated with allergy (Kurup et al., 2000). This mechanism has been linked to the occurrence of rhinitis and asthma (Samson et al., 1994). The prevalence estimates of IgE-mediated mold allergy vary considerably (5% - 50%) in different populations (Husman, 1996). In the general population, the prevalence of fungal allergy has been estimated to be 6%, whereas among atopic individuals it can be as high as 20% to 30% (Kurup et al., 2000). IgE-mediated allergy can explain only part of the observed symptoms and diseases. Taskinen and co-workers (1997, 1999, 2001) have shown that fungal allergy, as assessed by IgE measurements or skin tests was rare, although the symptoms and diseases were increased in moisture-damaged environments. Some fungi, especially *Stachybotrys chartarum* can trigger histamine release from human leukocytes by non-IgE-mediated mechanisms (Larsen et al., 1996). Thus, the fungal exposure may cause histamine-mediated symptoms also in non-sensitized population.

Another immunological reaction caused by microbial exposure is the immune complex-mediated (Type III) mechanism. This has been associated with extrinsic allergic alveolitis (i.e. hypersensitivity pneumonitis) and humidifier fever (Samson et al., 1994; Husman, 1996). The cell-mediated (Type IV) mechanism has also been reported to be provoked by fungal exposure (Samson et al., 1994; Tomee and van der Werf, 2001). The diagnosed cases of these

two latter types of allergies have been reported only occasionally to be connected with exposure in moisture-damaged buildings, but possibly this is partly due to underdiagnosis (Husman, 1996).

Immunosuppression and infections

In addition to immunostimulation or hyperreactivity, exposure to indoor air microbes or their products has been suspected to cause immunosuppression. The increased frequency of infections among occupants in moisture-damaged buildings suggests that host defense mechanisms have been impaired. Possible mechanisms include toxic effects on ciliated cells in the airways and subsequently impaired particle clearance (Pieckova and Jesenska, 1996, 1998), and toxic effects on other cells of the immune system (e.g. lymphocytes and alveolar macrophages). Several microbial products, such as trichothecene mycotoxins and products of actinobacteria have immunosuppressive potential (Pestka and Bondy, 1990; Ochiai et al., 1993; Wallemacq and Reding, 1993; Sorenson, 1999). The increased microbial burden and abnormal microbial composition in the buildings (e.g. opportunistic pathogens) may cause infections in individuals with an impaired host defense or poor health. Individuals at increased risk include immunocompromised patients, and patients suffering from respiratory diseases like chronic obstructive pulmonary disease. For example, *Aspergillus* species, especially *A. fumigatus* may cause pulmonary aspergillosis with different degrees of severity (Tomee and van der Werf, 2001). The most harmless manifestation of pulmonary aspergillosis, is "non-pathogenic saprophytic colonization" which is common even in healthy individuals. This type of microbial colonization should not cause tissue damage, and the healthy host will recover without treatment. In aspergilloma, the mycetoma (fungus ball) grows as a saprophyte in a preformed and poorly drained lung space. Hypersensitivity-induced aspergillosis includes *Aspergillus* asthma, allergic bronchopulmonary aspergillosis, and extrinsic allergic alveolitis. The most severe manifestation of aspergillosis is invasive aspergillosis, which is a generalized fungal infection.

Autoimmunity

Exposure to microbes in moisture-damaged buildings may also cause autoimmune reactions and diseases. An increased incidence of rheumatic diseases and aching joints have been reported in the occupants of moisture-damaged buildings (e.g. Platt et al., 1989; Roponen et

al., 2001). Microbes can induce autoimmunity, i.e. a direct immune response against host. Activation and subsequent clonal expansion of autoreactive lymphocytes (the cells that recognize and react against self-molecules) is a critical step in the pathogenesis of autoimmune disease (Wucherpfennig, 2001). Microbes can activate autoreactive T and B lymphocytes via several mechanisms (Table 3).

Table 3. Mechanisms which may lead to autoimmunity after microbial exposure (modified from Wucherpfennig, 2001).

- **Mechanisms based on microbial products or structures**
- Molecular mimicry. Sufficient homology between microbial immunogenic peptides and self-peptides that leads to activation of autoreactive lymphocytes.
- Microbial superantigens. Microbial molecules that induce uncontrolled stimulation and an inappropriate T cell response. Subpopulation of these cells may be autoreactive.

- **Inflammation associated mechanisms**
- Enhanced processing and presentation of autoantigens. During inflammatory process antigen processing and presentation are enhanced by antigen presenting cells recruited to the site of inflammation leading to activation of autoreactive lymphocytes.
- Bystander activation. Inflammation may increase the proliferation of previously activated autoreactive lymphocytes.

One possibility is that the cells do not recognize self-molecules, but instead persistent microbes or microbial antigens (structures/molecules) that have been carried along migrating phagocytes to the synovial tissues in the joints during long-term exposure. This has been suggested to be another mechanism, in addition to recognition of self molecules, contributing to the development of reactive arthritis (Wucherpfennig, 2001). DNA and RNA of *Chlamydia*, which is an intracellular bacterium, have been frequently detected from the synovial membrane or fluid during reactive arthritis. Very little is known about the possible role of microbial contaminants and mechanisms of autoimmune reactions in moisture-damaged environments.

Cytotoxicity

Microbial toxins can cause cytotoxicity which may be mediated via either apoptotic or necrotic mechanisms (Paananen et al., 2000; Yang et al., 2000; Etzel, 2002). However, other effects may occur at lower exposure levels not sufficient to induce cell death. For example, the mitochondrial toxin valinomycin produced by *Streptomyces griseus* reduced the natural killer (NK) cell-mediated cytotoxicity and cytokine production at a lower concentration than

that causing apoptotic cell death (Andersson et al., 1998; Paananen et al., 2000). Cytotoxic effects may also be directed against other cells, including leukocytes (e.g. alveolar macrophages, lymphocytes) and epithelial cells, and disturb both innate and adaptive host cell responses.

Neurotoxicity

The symptoms like fatigue and difficulties in concentrating (e.g. Koskinen et al., 1999b, Johanning et al., 1996) point to effects on the central nervous system. The products of *Fusarium* (fumonisin B_1, deoxynivalenol), *Aspergillus* (ochratoxin A) and *Penicillium* (ochratoxin A, verrucosidin) species have been shown to possess neurotoxic potential (Rotter et al., 1996; Belmadani et al., 1999; Kwon et al., 2000; Nunez et al., 2000). Also compounds that enhance neurite (axon and dendrite) outgrowth from neuronal cells have been isolated from fungal cultures (Nozawa et al., 1997).

Irritation

Microbial products such as microbial volatile organic compounds (MVOC's) may cause irritation via neurogenic mechanisms. When chemosensitive receptors are activated in the airways and alveoli, the subsequent local release of neuropeptides (e.g. substance P and neurokinin A) may provoke neurogenic inflammation (Damgård Nielsen et al., 1995). Some of the neuropeptides can also affect innate immune responses. Substance P increases phagocytosis and enhances neutrophil accumulation and reactivity by facilitating the actions of other inflammatory mediators (Mathison et al., 1993; Damgård Nielsen et al., 1995).

Genotoxicity

Some microbial toxins are genotoxic and carcinogenic. Inhalation exposure to a mycotoxin produced by *Aspergillus flavus*, aflatoxin, has been associated with lung or colon cancer (Olsen et al., 1988; Sorenson, 1999). Naturally occurring aflatoxins are human carcinogens, and several other toxins and secondary metabolites including adriamycin, daunomycin (i.e. daunorubicin) and streptozotocin (produced by streptomycetes), sterigmatocystin and ochratoxin A (*Aspergillus* and *Penicillium*), and fumonisins B_{1-2}, and fusarin C (*Fusarium*) have been considered risk factor for cancer (IARC, 1987; 1993). However, no

epidemiological or experimental data have been published concerning exposure in moisture-damaged buildings and risk of cancer.

Reproductive toxicity

Adverse effects during pregnancy are also possible. An association between occupational exposure to mycotoxins and late-term abortions has been proposed (Kristensen et al., 1997), and mycotoxins have been reported to cause reproductive toxicity in animals (Korpinen, 1974). Oral exposure to *Stachybotrys chartarum* during the early phase of pregnancy provoked reproductive toxicity in mice (Korpinen, 1974). Very little is currently known about these forms of toxicity in moisture-damaged indoor environments.

2.4 Host defense with special reference in lungs

2.4.1 Physiological defenses in lungs

The lungs possess a variety of structural, mechanical, chemical and cellular strategies to guarantee proper function and form of the airways for effective gas exchange (Whitsett, 2002). A major part of larger particles (> 5 µm) is removed by filtration from the inhaled air in the upper airways (Zhang et al., 2000). Most of the microbes or spores in the indoor air of moisture-damaged buildings are at the size-range which allow them to deposit also to the alveolar level (Hyvärinen et al., 2001b). Even the relatively large spores of *Stachybotrys chartarum* (aerodynamic diameter approximately 4 - 5 µm) can penetrate the alveoli (Sorenson, 1987). The physiological defense mechanisms in the lungs include the coughing reflex and the ability to evoke bronchoconstriction (Sant'Ambrogio and Widdicombe, 2001). Several cell types are important for proper host defense responses in the lungs. The epithelial cell types include ciliated, non-ciliated bronchiolar (Clara cells), mucus producing (goblet cells), neuroepithelial cells, and type II and type I alveolar epithelial cells (Whitsett, 2002). In addition, phagocytic cells are normally found in the lungs.

2.4.2 Innate host defense

When microbial contaminants enter the lungs, they will first encounter the innate host defense. Epithelial cells are an effective physical barrier which can also produce mucus, and

transport the adhered contaminants to the pharynx via cilia movement (Zhang et al., 2000; Knowles and Boucher, 2002). The transported mucus and contaminants are either swallowed into the gastrointestinal tract or expectorated. In addition to the mechanical clearance, respiratory secretions in the airways have antimicrobial effects (Ganz, 2002). These secretions are produced by several cell types, including epithelial cells, especially goblet cells, submucosal glands, and resident as well as recruited phagocytes. Moreover, transudation and transport of proteins from circulation into the airways can occur. Important secretory cells in the distal airways include alveolar epithelial cells, such as Clara cells and type II epithelial cells. The broad antimicrobial effects of respiratory secretions are mainly attributable to cationic polypeptide components, such as the cell-membrane degrading enzyme lysozyme, the iron-chelating protein lactoferrin, secretory leucoprotease inhibitor, neutrophil and epithelial defensins which permeabilize cell-membranes, and cathelicidins which have a diverse range of antimicrobial activity (Ganz, 2002; Ramanathan et al., 2002).

Agents that reach the alveolar level and which become deposited into the alveoli come into contact with the alveolar lining layer (ALL) (McCormack and Whitsett, 2002). ALL is a thin aqueous film containing pulmonary surfactant. Its components, including pulmonary collectins, also participate in the innate host defense (LeVine and Whitsett, 2001; McCormack and Whitsett, 2002). The primary components of antimicrobial defense in alveolar region for naive host are resident alveolar macrophages and the protein components present also in respiratory secretions. Alveolar macrophages are phagocytic cells that decontaminate the alveolar walls. They are activated by innate immune receptors such as CD14 and Toll-like receptors (TLRs), which detect lipopolysaccharide (LPS) molecule on the surface of Gram-negative bacteria. Phagocytosis is triggered by several different kinds of receptors (e.g. mannose receptor, macrophage scavenger receptor) (Greenberg and Grinstein, 2002). Endocytic pattern-recognition receptors are located on the surface of the phagocytes, and they can identify microbial structures and promote phagocytosis (Medzhitov and Janeway, 2000). If a microbe has been encountered previously, the phagocytosis can also be triggered by Fc-receptors after the microbe has been opsonized by specific antibodies. Phagocytized microbes are destroyed in phagolysosomes by the action of lethal radicals and hydrolytic enzymes (Greenberg and Grinstein, 2002). Innate pattern-recognition receptors have been functionally divided into two more classes in addition to endocytic receptors: secreted and signaling receptors (Medzhitov and Janeway, 2000). Secreted pattern-recognition receptors, such as mannan-binding lectin are opsonins that bind to the microbial structures and flag the

microorganism to the phagocytes and the complement system for recognition. Signaling pattern-recognition receptors activate signal-transduction pathways after recognition of microbial structures leading to the induction of inflammatory cytokine production. The Toll-like receptor family is an example of signaling pattern-recognition receptors. Inflammatory mediators, such as cytokines produced by activated alveolar macrophages, may provoke early induced non-adaptive inflammatory responses, e.g. recruit neutrophils into the airways or induce production of acute-phase proteins in the liver (Janeway and Trawers, 1997).

The complement system, a cascade of serum proteins, can attack invading microorganisms. The complement system activated by antibody-independent alternative (initiation by C3b protein) or lectin-mediated pathway is one of the innate host defense mechanisms in the lungs (Medzhitov and Janeway, 2000). Moreover, during the activation of the complement cascade, inflammatory mediators, such as C5a, C4a, and C3a, are released (Delves and Roitt, 2000a). These mediators can recruit and activate neutrophils, and also trigger the release of histamine from mast cells.

Since the innate immunity responds more rapidly than the adaptive immunity, it is crucial also for the antimicrobial immunity in the lungs (Medzhitov and Janeway, 2000). Even though the diversity of the recognition patterns in the innate receptors is nowhere near the repertoire of antigen specific receptors in the adaptive host defense, innate receptors still effectively recognize common and conserved structural patterns in microbes. Moreover, properly functioning innate responses are also important in initiating and directing the responses of the adaptive host defense. Innate host defense responses do not adapt during the exposure. The response neither intensifies during the later exposure nor does it possess memory.

2.4.3 Adaptive host defense

Lymphocytes, fundamentally divided into T and B cells, are crucial cells for the adaptive immunity (Delves and Roitt, 2000a). In addition, there is increasing evidence of unconventional lymphocytes that do not clearly belong to traditional T or B cell types (Tabata et al., 1996; Penttilä et al., 1998; Phyu et al., 1999; Zuckermann, 1999). However, their functions are not well understood.

T cells include $CD4^+$ and $CD8^+$ subpopulations (Delves and Roitt, 2000b). $CD4^+$ T cells are mainly cytokine-secreting helper cells, whereas $CD8^+$ cells function as cytotoxic killer cells (Tk cells). Helper T cells have been further divided into two major subpopulations, Th1 and Th2, based on their cytokine profiles (Mosmann and Sad, 1996). Type 1 helper cells preferentially produce interferon-γ (IFNγ) and IL-2 but not IL-4, IL-5 or IL-6. Type 2 T cells produce, for example, IL-4, IL-5, IL-6 and IL-10 but not IFNγ or IL-2 (Delves and Roitt, 2000b; Moore et al., 2001). A simplified view is that Th1 cytokines enhance cell-mediated immunity for example by activating macrophages or T cell-mediated cytotoxicity, and Th2 cytokines stimulate B cells to produce antibodies.

Cytotoxic T cells can kill cells that introduce foreign peptides from their cytoplasm via major histocompatibility complex (MHC) class I -molecules (Delves and Roitt, 2000b). These peptides are a sign of an intracellular infection for Tk cells. Killing can be executed by two mechanisms: A) Tk cells can secrete perforins which produce pores in the cell membrane of the target cell, and then proteolytic enzymes, granzymes enter into the target cell from the Tk cell. Granzymes induce apoptotic cell death. B) Tk cells can also induce apoptosis via a Fas-mediated pathway. $CD8^+$ cells presumably include other subpopulations than traditional Tk cells, as well as Th cells (Moore et al., 2001).

Plasma cells, differentiated from B cells after antigen recognition and the cell activation, are responsible for antibody production (Delves and Roitt, 2000b). Antibodies can be self-protective if they inhibit direct contact between microbe or toxin and the corresponding cell receptor (neutralization). However, in most cases, antibodies activate and direct other host defense components, such as complement, against the microbial invader. Antibodies opsonize microbes to facilitate phagocytosis, form large antigen-antibody complexes (agglutination), and induce antibody-dependent cellular cytotoxicity (Moore et al., 2001). B cells also act as antigen presenting cells (Delves and Roitt, 2000b). Moreover, so called T-independent antigens, like LPS in the bacterial cell wall, directly activate B cells without the need for T cell help (Moore et al., 2001).

Activation of the adaptive immunity requires a complex interplay between lymphocytes and other cell types (Moore et al., 2001). For example, antigen presenting cells, such as dendritic cells and macrophages are essential for the activation of T lymphocytes (Delves and Roitt, 2000b). In addition to the appropriate receptor for antigen recognition on T cells,

costimulatory signals are needed for activation. These signals include cell surface receptor-ligand interactions between the T cell and antigen-presenting cell. Moreover, cytokines such as IL-1, IL-6, IL-12, IL-18 and TNFα can provide costimulatory signals (Delves and Roitt, 2000b; Giacomini et al., 2001). Interestingly, antigen recognition without the appropriate costimulatory signal leads to T cell anergy or apoptosis. T cell activation and responses are also regulated by inhibitory signals, which may be either cell surface molecule interactions or cytokine (e.g. IL-10) -mediated.

The adaptive host defense possesses a memory, which enables a faster response against the antigen at subsequent encounters. Adaptive immune responses depend greatly on the microbe and its immunogenic structures, the genetic background of the host, and the dose and route of exposure (Moore et al., 2001). However, induction of these responses requires a substantially longer time than that needed for innate and non-adaptive responses (Medzhitov and Janeway, 2000).

2.5 Inflammation

2.5.1 General

Inflammation (or the inflammatory response) is a response to different stimuli including exposure to chemicals or microbes. As such, inflammation is a rather non-specific response, as described with the latin terms "calor, rubor, tumor, dolor" (heat, redness, swelling, and pain). However, there can be great differences in the ability of different substances to induce inflammation. Cellular responses against different bacterial species vary markedly: alveolar macrophages are capable of defending the lungs against *Staphylococcus* (Rehm et al., 1980). Neutrophils, instead, seem to be crucial for protection against *Pseudomonas*. Subsequently, the inflammatory responses (mediators, recruited cells) evoked by microorganisms can be somewhat different.

In the acute inflammatory response, cells of the immune system move into the site of inflammation (Delves and Roitt, 2000a). Inflammatory mediators produced by affected cells contract local blood vessels and increase their permeability, leading to fluid extravasation. Expression of adhesion molecules on the vascular endothelium is up-regulated, and the neutrophils rolling on the vessel wall adhere to these molecules and pass through the wall. A

chemotactic gradient of chemoattractive mediators directs inflammatory cells to the affected site.

Microbial antigens entering the body through the mucosal surface will activate cells in mucosa-associated lymphoid tissue (Delves and Roitt, 2000b). In the lungs, this kind of lymphoid tissue is bronchus-associated lymphoid tissue (BALT). BALT is present in the lungs of children and adolescents, and in adult lungs in different diseases, but not in the healthy lungs of adults (Tschernig and Pabst, 2000). Responses to intranasal and inhaled antigens occur in palatine tonsils and adenoids (Delves and Roitt, 2000b). Moreover, microorganisms in tissue can induce responses in the local lymph nodes. The responses to antigens that have spread to blood circulation are usually initiated in the spleen.

2.5.2 Inflammatory cells

In the generation of inflammation, cells located at the site of exposure are in a crucial position. Important inflammatory cells include macrophages, neutrophils, lymphocytes and eosinophils. During exposure, the responses of these cells to a major extent determine the outcome of the inflammatory response.

Macrophages

Alveolar macrophages, within the alveolar surfactant film at the interface between air and lung tissue, possess high phagocytic and microbicidal potential (Lohmann-Matthes et al., 1994). In the normal resting animal, alveolar macrophages represent more than 90% of the lavagable cells in the lungs. They are the most important pulmonary macrophages with respect to microbial killing. These cells are capable of producing reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-). These radicals have an important role in both intracellular and extracellular defense against microorganisms. Moreover, alveolar macrophages can produce many cytokines such as TNFα, IL-1, IL-6, IL-8, IL-12, IL-18, interferons, defencins, and nitric oxide (Lohmann-Matthes et al., 1994; Moller et al., 1996; Vankayalapati et al., 2000). These mediators are also involved in the antimicrobial activity of the cells. In addition, alveolar macrophages can produce a wide variety of other products such as enzymes (e.g. lysozyme and collagenases), biologically active lipids (e.g. thromboxane A_2 (TXA_2), prostaglandins, and leukotrienes),

antiproteases and other inhibitors, fibronectin, complement components (e.g. C2 and C4), binding proteins (e.g. transferrin), free fatty acids, antioxidants (glutathione), and coagulation factors (Sibille and Reynolds, 1990).

Pulmonary macrophages also include interstitial macrophages, which are located in the lung connective tissue (Lohmann-Matthes et al., 1994). These cells have partly different functional properties than alveolar macrophages. Even though Fc-receptor-mediated phagocytosis is equally effective, Fc-receptor-independent phagocytosis and the capability to produce inflammatory mediators and oxygen radicals is weaker in the interstitial than in alveolar macrophages. Instead, antigen presentation via MHC II molecules is more effective in interstitial cells. The alveolar and interstitial macrophages derive from blood monocytes recruited into the lungs, and they can also locally proliferate in the alveoli or interstitium.

Intravascular macrophages are located on the endothelial cells in the blood capillary lumen. They are highly phagocytic and thus differ from monocytes. These cells are believed to remove foreign and damaging material from the bloodstream. This type of pulmonary macrophages has been observed in humans but not in rodents (Lohmann-Matthes et al., 1994).

Dendritic cells

Dendritic cells are located in small numbers in the lung interstitial tissue (Lohmann-Matthes et al., 1994). Their role in tissues is to capture antigens and microbes and after being activated leave the peripheral tissue and locate into the local lymph node, where they function as antigen-presenting cells.

Neutrophils

Chemokines released by alveolar macrophages recruit monocytes and neutrophils from the circulation to the site of inflammation (Stockley, 1995). Neutrophils can produce a variety of different substances, such as lipids (leukotrienes, platelet activating factor (PAF), TXA_2), cytokines (IL-1β, IL-6, IL-8, TNFα), proteases (elastase, collagenase), microbicidal products (lactoferrin, myeloperoxidase, lysozyme), ROS, and NO (Sampson, 2000). In addition to being important phagocytic cells against respiratory pathogens, neutrophils may also have destructive effects on lung cells and interstitial tissues (Stockley, 1995; Sampson, 2000). One

culprit of these harmful effects is the proteolytic enzyme, neutrophil elastase, which is released when the activated neutrophil phagocytizes microbes (Stockley, 1995). Neutrophil elastase causes excess mucus production, inhibits ciliary function, impairs phagocytic microbial clearance, and stimulates chemokine production. These effects may delay microbial clearance, and cause excess neutrophil recruitment into the lungs. Moreover, elastase can activate eosinophils and mast cells (Sampson, 2000).

Lymphocytes

Lymphocytes (see 2.4.3) are crucial when the capacity of the innate host defense is overwhelmed and also in hypersensitivity reactions.

Eosinophils

Eosinophils are bone marrow derived granulocytes, which are present in tissues and blood in very low numbers (Weller, 1997). However, their number is increased in allergic diseases and parasitic (helminth) infections. Eosinophils have been considered to be especially important cells in asthmatic airway inflammation (Sampson, 2000). They can produce cytotoxic basic proteins (e.g. eosinophil cationic protein, eosinophil peroxidase, major basic protein) and lipid mediators including cysteinyl leukotrienes that provoke airflow obstruction and bronchial epithelial damage. Moreover, eosinophils can produce PAF and TXA_2.

Mast cells and basophils

Mast cells and basophils are important cells in IgE-mediated allergic reactions and the defense against certain parasitic infections, since they bind IgE antibodies efficiently with specific receptors and subsequently release biologically active mediators such as histamine and cytokines (Abraham and Arock, 1998; Kurup et al., 2000). The activated cells can also produce leukotrienes and prostaglandins. In addition to being involved in IgE-mediated adaptive immune responses there is increasing evidence that these cells, especially mast cells, contribute also to innate immunity (Abraham and Arock, 1998). Microbes can activate these cells also via antibody-independent mechanisms.

2.5.3 Cytokines

Cytokines are small proteins (8 - 40,000 Da) produced by nearly all nucleated cells, which also respond to them (Dinarello, 2000). These proteins mediate their effects via specific cell surface receptors in immune or non-immune cells, and they are necessary for the full development of innate host defense responses and transition to adaptive immunity (Strieter et al., 2002). Cytokines can have both paracrine and autocrine effects (Kelley, 1990), and they can provoke (e.g. via TNFα and IL-1) or suppress inflammation (e.g. via IL-10) (Dinarello, 2000). Chemokines can recruit neutrophils and other leukocytes from blood to the site of inflammation (Sabroe et al., 2002). Moreover, these cytokines are important in regulating leukocyte trafficking in normal tissue homeostasis. Out of the wide cytokine network, two cytokines, TNFα and IL-6, which have been analyzed in this study are described in more detail.

Tumor necrosis factor α

TNFα is a 17-kDa polypeptide. Its responses are mediated through two distinct receptors, a constitutive receptor (TNFR1) and an induced (TNFR2) receptor (Luster et al., 1999). TNFα can stimulate the synthesis of other mediators that then regulate cell differentiation and growth, antiviral activity, immunomodulation and inflammation. Moreover, it possesses cytostatic and cytotoxic activities, but these effects are associated primarily with transformed cell lines and tumor cells, and to a lesser extent with normal cells. TNFα, for example, is mitogenic to lymphocytes, and it increases the activity of macrophages/monocytes, neutrophils, lymphocytes, natural killer (NK) cells, and endothelial and epithelial cells (Barnes et al., 1998; Luster et al., 1999). Moreover, it stimulates the production of several hormones, such as ACTH and thyroid stimulating hormones (Luster et al., 1999).

TNFα regulates the production of many other inflammatory mediators such as IL-1, IL-6, IL-8, granulocyte macrophage-colony stimulating factor, and some adhesion molecules (Barnes et al., 1998; Luster et al., 1999). Thus, many of its apparent effects are not directly TNFα-mediated (Luster et al., 1999). TNFα may also directly damage pulmonary vascular endothelium and subsequently cause capillary leakage. TNFα seems to have a dual role in the lungs. Low levels of the cytokine are present in the normal lungs, and under such conditions it

may also have a protective role via induced expression of a potent antioxidative enzyme, superoxide dismutase. However, elevated levels of TNFα in lung fluids have been associated with many inflammatory lung diseases, such as chronic bronchitis, prolonged cough, bacterial pneumonia, adult respiratory distress syndrome, cystic fibrosis and asthma (Dehoux et al., 1994; Barnes et al., 1998; Jatakanon et al., 1999; Luster et al., 1999). Moreover, elevated levels of this cytokine have been observed during pathological conditions resulting from environmental exposure, including pollutant-induced inflammatory disease (e.g. caused by grain and swine dust, coal-mine dust, quartz and asbestos), hypersensitivity pneumonitis and occupational asthma (Vanhee et al., 1995; Driscoll et al., 1997; Von Essen, 1997; Wang et al., 1997; Luster et al., 1999). Alveolar macrophages have been suspected to be the major source of TNFα in the lungs, but it is produced by several cell types, including lung endothelial and epithelial cells, T lymphocytes and mast cells (Barnes et al., 1998; Luster et al., 1999).

Interleukin-6

IL-6 is a glycoprotein mediator which is produced by a wide variety of cells including monocytes/macrophages, T and B cells, fibroblasts, mast cells, endothelial cells, and tumor cells (Kelley, 1990; Van Snick, 1990; Barnes et al., 1998). It can induce B cell differentiation to antibody forming plasma cells, and augment antibody production (Hirano et al., 1986; Kelley, 1990; Barnes et al., 1998). It is also involved in T cell growth, differentiation, and activation (Barnes et al., 1998). It increases T cell proliferation, presumably via increased IL-2 receptor expression (Van Snick, 1990). IL-6 production is induced for example by a Gram-negative bacterial cell wall component, LPS, and other inflammatory mediators such as TNFα and IL-1. It has synergistic effects with IL-1. On the other hand, IL-6 may also have anti-inflammatory effects, since it decreases TNFα and IL-1 production in macrophages (Barnes et al., 1998). It may also decrease neutrophil influx into the airways. IL-6 is produced in asthma and bacterial pneumonia (Dehoux et al., 1994; Barnes et al., 1998). Exposure to organic or mineral dusts (grain and swine dusts, and coalmine dust) increase the cytokine level in the lungs (Vanhee et al., 1995; Von Essen, 1997; Wang et al., 1997). There is some evidence that excessive production of IL-6 may induce polyclonal B cell activation leading to hypergammaglobulinemia and autoantibody production (Van Snick, 1990). Moreover, overproduction of IL-6 has been implicated in some localized proliferative diseases.

IL-6 and TNFα have also systemic effects. IL-6 in blood induces production of the acute phase proteins (e.g. C-reactive protein, serum amyloid A, α_1-chymotrypsin, and fibrinogen), whereas it down-regulates albumin production in the liver (Kelley, 1990). TNFα also induces production of the proteins, even though it does not affect as many proteins as IL-6 (Van Snick, 1990). Both TNFα and IL-6 are endogenous pyrogens that can cause fever (Luheshi and Rothwell, 1996).

2.5.4 Nitric oxide

Nitric oxide is a free radical and an important mediator, which is produced from L-arginine via an enzymatically catalyzed process (Singh and Evans, 1997). The enzymes that produce NO have been divided into two classes depending on their special features: constitutive and inducible NO synthases. Constitutive forms (cNOS) include neuronal NOS (nNOS) and endothelial NOS (eNOS) that are continuously expressed in these cells. These enzymes can produce relatively small amounts of NO, and this NO production is involved for example in non-adrenergic and non-cholinergic (NANC) neurotransmission and regulation of blood circulation. Expression of the inducible form of the enzyme (iNOS) is evoked only by the appropriate stimuli, but iNOS can produce relatively high amounts of the radical. NO produced by iNOS is involved in host defense against infection and inflammatory diseases of the airways (Barnes et al., 1998). At least macrophages and epithelial cells can express iNOS in the lungs (Asano et al., 1994; Singh and Evans, 1997). NO has a short half-life, and it decomposes rapidly to nitrite (NO_2^-) and nitrate (NO_3^-) (Singh and Evans, 1997). However, it can also combine with the superoxide anion (O_2^-) and form peroxynitrite ($OONO^-$), which has toxic effects on many molecules, including nucleic acids, lipids and proteins.

Recently it has been shown that human macrophages produce NO in several inflammatory conditions, including tuberculosis, rheumatoid arthritis and malaria (Fang and Vasquez-Torres, 2002). NO plays a role in antimicrobial host defense also against extracellular pathogens in human as well as in murine macrophages (Hickman-Davis et al., 2002; Fang and Vasquez-Torres, 2002). Inducible NOS expression has been detected in human alveolar macrophages and airway epithelial cells during interstitial pneumonia in adults (Lakari et al., 2002). Increased concentrations of NO in exhaled air, representing the fraction produced in

the lower airways, have been observed in several inflammatory diseases, including asthma, lower respiratory tract infection, bronchiectasis and chronic obstructive pulmonary disease (Alving et al., 1993; Kharitonov et al., 1994; Kharitonov et al., 1995; Maziak et al., 1998). Exhaled NO level can be elevated also during IgE-mediated allergy (Adisesh et al., 1998; Henriksen et al., 1999).

3 AIMS OF THE PRESENT STUDY

The overall aim of the study was to investigate experimentally *in vivo* the inflammatory and toxic responses induced by certain microbes isolated from indoor air of moisture-damaged buildings. The effects of *Streptomyces californicus* (I and V), *Mycobacterium terrae* (II), *Aspergillus versicolor* (III), and *Penicillium spinulosum* (IV) were studied after airway exposure in mice.

The more specific goals of the present study are:

(I - IV) to compare the effects and responses caused by microbial exposure in the lungs.

(I - V) to evaluate whether the airway exposure can provoke systemic effects.

(V) to compare the responses induced by *S. californicus* after repeated or single dose of the microbe.

4 MATERIALS AND METHODS

4.1 Animals (I - V)

SPF-quality (free of specific pathogens) male NIH/S mice were used in all studies. The animals were obtained from the breeding colony of the National Public Health Institute, Division of Environmental Health, Kuopio, Finland. They were transferred from the barrier unit to a conventional animal room and housed singly in metal cages on aspen wood chips (FinnTapvei, Finland) one week before the experiments. Animals received water and food [R36 Maintenance Diet for rats and mouse (Lactamin, Stockholm, Sweden)] *ad libidum*. The mice were on a 12-h light/dark rhythm (7 a.m. to 7 p.m.) at the average room temperature of 21-22°C, and relative humidity ranging from 26-47%.

4.2 Microbes (I - V)

Four microbial species were selected: 1) *Streptomyces californicus*, 2) *Mycobacterium terrae*, 3) *Aspergillus versicolor*, and 4) *Penicillium spinulosum* (Table 4). These species were selected, since they are typically found microbial species in indoor air of moisture-damaged buildings.

Table 4. The microbial species studied.

Species	Microbial type	Particle size
Streptomyces californicus	Gram-positive bacterium	~ 1 µm spore (Reponen et al., 1998)
Mycobacterium terrae	mycobacterium	~ 1 µm cell (microscopy)
Aspergillus versicolor	fungus	2.0-3.5 µm spore (Guarro et al., 1995)
Penicillium spinulosum	fungus	3.0-3.5 µm spore (Guarro et al., 1995)

The streptomycetes, *Aspergillus* and *Penicillium* species have been frequently observed in the indoor air of moisture-damaged buildings, and they have been considered to be moisture indicator microbes (Samson et al., 1994). *P. spinulosum* is commonly observed also in other buildings, but in the damaged buildings fungal spore numbers are increased. Mycobacteria are

a less known microbial species in indoor air exposures in the moisture-damaged buildings (Andersson et al., 1997). These microbes have had different potential to cause cytokine production and/or cytotoxicity in mouse macrophages *in vitro* (Hirvonen et al., 1997a, 1997b, 1997c; Huttunen et al., 2000; Huttunen et al., in press).

All the microbial strains were isolated, identified and cultured in the Laboratory of Environmental Microbiology, National Public Health Institute, Kuopio, Finland. A strain of *Streptomyces californicus* (I and V), a mesophilic Gram-positive bacterium, was isolated from the indoor air of a moldy building, as described by Hyvärinen and co-workers (1993). It was identified by the DSM identification service (DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). *Mycobacterium terrae* (II) was recovered from the indoor air of a moldy building (Rautiala *et al*., 1996). The isolate was identified by gas liquid chromatography of the fatty acids, fatty alcohols and mycolic acid cleavage products, and by its growth and biochemical characteristics. The isolate was tested to be negative for a commercially available DNA probe specific for *Mycobacterium avium* complex (AccuProbe, Gen-Probe Inc., San Diego, CA) (Torkko *et al.*, 1998). *Aspergillus versicolor* (III) and *Penicillium spinulosum* (IV) were isolated from the indoor air of a moldy building. The strains were identified morphologically, and the identification was verified by the CBS identification service (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands). All the microbes were cultured on standard laboratory media at appropriate temperature and for an adequate time (I - V). The microbes were collected, and spore/cell numbers were counted under epifluorescence or a light microscope. The Gram-negative bacterial lipopolysaccharide (LPS) (Escherichia coli, serotype 0111:B4, Sigma, USA) was used as a reference agent for biological responses in the model (I).

The doses of the microbes and the time points for collection of samples (Table 5) were selected on the basis of the results obtained in our preliminary and completed *in vivo* experiments, and the previous *in vitro* studies. The total volumetric load of the doses, i.e. physical particle sizes of the spores and cells also affected the dose selection. High volumetric doses may cause non-specific effects by overloading macrophages (Morrow et al., 1992), and such effects are not relevant for lower dose exposure.

Table 5. The doses and schedules for sampling in A) single dose studies and B) the repeated dose study.

Strain	Dose-response experiments		Time-course experiments	
	Dose (spores or cells/animal)	**Sampling time**	**Dose (spores or cells/animal)**	**Sampling times**
A) Single dose experiments				
S. californicus (I)	2×10^7	24 h	1×10^8	3h, 6h, 24h, 3d, 7d
	$\mathbf{1 \times 10^8}$ [a]	"		
	3×10^8	"		
M. terrae (II)	1×10^7	"	1×10^8	6h, 24h, 3d, 7d, 14d, 21d, 28d
	5×10^7	"		
	$\mathbf{1 \times 10^8}$	"		
A. versicolor (III)	1×10^5	"	5×10^6	6h, 24h, 3d, 7d, 14d, 21d, 28d
	1×10^6	"		
	$\mathbf{5 \times 10^6}$	"		
	1×10^7	"		
	1×10^8	"		
P. spinulosum (IV)	1×10^5	"	5×10^6	6h, 24h, 3d, 7d, 14d, 21d, 28d
	1×10^6	"		
	$\mathbf{5 \times 10^6}$	"		
	1×10^7	"		
	5×10^7	"		
B) Repeated dose experiment			**Dosing and sampling schedule**	
S. californicus (V)	2×10^3		- The dose on days 0, 7, 14, 21, 28, and 35.	
	2×10^5		- Sampling 24 hours after the last dosage.	
	2×10^7			

[a] Equal volumetric doses in dose response experiments have been indicated in bold font.

4.3 Instillation (I - V)

Animals were anesthetized with the inhalation anesthetic, sevoflurane (Sevorane®, Abbot, Illinois, USA), and exposed either to the microbial spores/cells or HBSS (carrier control, Gibco, UK) by intratracheal instillation. To deliver the dose, an anesthetized mouse was placed at a 66° upward prone posture and the incisors were placed on a wire. The dosing was performed under visual control by using a cold-light source (KL 1500electronic, Schott,

Germany) placed against the throat. The nostrils were blocked, the tongue was pulled out, and the dose (50 μl/animal) was delivered onto the vocal folds with a Finn-pipette tip.

4.4 Surgical preparation and sample collection

4.4.1 Anesthesia and blood samples (I - V)

After the exposure period, the animals were anesthetized with an i.p. injection of pentobarbital and exsanguinated by cardiac puncture. Cardiac puncture was done after opening the abdominal cavity and removal of the sternum. Blood was collected and serum separated (Capiject T-MG®, Terumo, MD, USA) for cytokine analyses.

4.4.2 Bronchoalveolar lavage, sample collection and cell counting (I - V)

The tracheas of the animals were exposed and cannulated with polyethylene tubing. At first, the lungs were lavaged with two portions of sterile modified HBSS (30 ml/kg), three times with each, and these two portions were combined. Thereafter, to get a greater number of cells, the lungs were further lavaged with six portions of HBSS as before, and these portions were combined together. Lavage fluids of the two sets were kept on ice. Cytospin was executed from the first set of lavages. Slides were fixed in methanol and stained with the May-Grünwald–Giemsa dye, and cell differentials were defined. Cells were separated from the first two lavages by centrifugation and the supernatant was centrifuged once more to discard all red blood cells and cell debris. Lactate dehydrogenase (LDH) concentrations were analyzed in fresh supernatants. The rest of the supernatants were frozen (-80°C) for analyses of TNFα, IL-6, albumin and total protein concentrations.

Cells of the first two lavages were resuspended in 200 μl of modified HBSS and the total cell number of each sample (cells/ml BALF) was counted by the Trypan blue exclusion method. The rest of the cell suspension was centrifuged, and after discarding the media, red blood cells were hemolyzed from the cell pellet by hypotonic shock, and the supernatant samples were collected for hemoglobin assay. Cells of the other set of lavages were centrifuged, the buffer discarded and red blood cells hemolyzed. The cells were combined with the surplus cells from the first set of lavages, pelleted and frozen for later Western blot analyses of iNOS.

4.4.3 Isolation of cells for flow cytometric analysis (V)

Inflammatory cells in the lungs, spleen and lymph nodes (mediastinal, axillary, brachial, and both superficial and deep cervical nodes) were analyzed by flow cytometry. The tissues were dissected, and homogenized to single cell suspension. Erythrocytes were hemolyzed by hypotonic shock from lung and spleen samples. At the end, the hematopoietic cell concentration (erythrocytes excluded) of each sample (cells/ml) was counted by the Trypan blue exclusion method, and the total cell number in each organ was calculated.

4.4.4 Dissection for histopathological analysis (I - V)

The lungs (filled with 10% phosphate buffered formalin), liver, spleen and lymph nodes (mediastinal (I - V), axillary (II - V), brachial (II - V), and both superficial and deep cervical nodes (II - V)) were preserved in 10% phosphate buffered formalin for histopathological evaluation.

4.5 Experimental protocols (I - V)

Both dose-responses and time-courses were studied (I - IV). In dose-response experiments, the animals were exposed to a single dose of microbes, and the responses were measured 24 h after the dosing. In the time-course experiments, the responses induced by a single dose at a one-dose level were studied at 5-7 different time points (Table 5). In the repeated dose study (V), the dose-response induced by the spores of *S. californicus* was investigated by exposing mice to six doses at a 7 day interval (at 3 dose levels beside the control) (Table 5).

4.6 Analyses

The parameters analyzed and their main pathophysiological functions/indications are listed in Table 6.

Table 6. Parameters and their main pathophysiological functions/indications.

Parameters	**Pathophysiological functions/indications**
Total cell number and cell differentials	Degree/state of inflammation in lungs
Cytokines (TNFα and IL-6)	Activation of immunological mechanisms (e.g. inflammation)
Nitric oxide	Initiation of inflammation, cell death
Total protein and albumin	Edema, tissue/cell damage
Hemoglobin in BALF	Inflammation, tissue damage
Lactate dehydrogenase (LDH)	Cell death
Histopathology	Degree/state of inflammation, structural damage
Lymphocyte subpopulations	Activation of immunological mechanisms, immunotoxicity
The comet response	DNA-damage/genotoxicity

4.6.1 Detection of cytokines (I - V)

Cytokines (TNFα and IL-6) were analyzed from BALF and serum by using the ELISA-method. Antibody pairs were from R&D Systems (Minneapolis, MN, USA) and analyses were performed according to the manufacturer’s instructions. Briefly, 96-well microtiter plates were coated with monoclonal capture antibody, and the cytokine in the samples and standards were allowed to adhere. In the analyses of BALF samples, the standards were diluted in Diluent, and in serum analyses in NIH/S mouse serum collected from animals of our stock. The biotinylated second antibody was used. Streptavidin conjugated horseradish peroxidase (HRP) was used as a detection reagent, and tetramethylbenzidine (TMB) solution as a substrate. The absorbances were measured by ELISA reader (iEMS Reader MF, Labsystems, Finland) at the wavelength of 450 nm.

4.6.2 Western blotting for iNOS (I - V)

Cells were lysed, released proteins were denatured, and samples, protein markers (Bio-Rad) and positive controls were subjected to sodium-dodecyl sulphate polyacryl amide gel electrophoresis. The protein amount in each sample was calculated after determination of the

protein concentration by DC Protein Assay (Bio-Rad, Hercules, CA, USA). Proteins were then electrophoretically transferred to a nitrocellulose (I) or PVDF (II - V) membrane. After non-specific binding was blocked with BSA, the membranes were incubated with primary antibody solution [0.1% Rabbit Anti-iNOS pAb (Transduction Laboratories, USA) in BSA] for 1 hour. Free primary antibody was removed by washings, and the membranes were incubated in alkaline phosphatase conjugated second antibody solution [0.1% AP-Goat Anti-Rabbit IgG (Zymax®, Zymed, CA, USA) in BSA] for 1 h. The membranes were washed again to remove free secondary antibody. Finally, the membranes were developed using 5-bromo-4-chloro-3-indolyl phosphate disodium (BCIP)/nitro blue tetrazolium (NBT) solution, and the reaction was stopped by rinsing the membranes in tap water.

4.6.3 Flow cytometric analysis (V)

Approximately 1.5×10^5 lung, spleen or lymph node cells were washed and resuspended in 2% fetal bovine serum (FBS) in HBSS. Nonspecific binding was blocked by CD16/CD32 monoclonal antibody (Fc Block™, PharMingen, CA, USA). Then the samples, except the controls, were stained by PE -conjugated CD45, CY-Chrome™ -conjugated CD3, and either FITC -conjugated CD25 or FITC -conjugated CD4 monoclonal antibodies (all obtained from PharMingen, CA, USA). Control cells were stained with anti-human monoclonal antibodies (PE -conjugated CD8 (Leu™-2a, Becton Dickinson, CA, USA), PerCP -conjugated CD3 (Leu™-4, Becton Dickinson), and FITC -conjugated anti-TCR-γ/δ-1 (Becton Dickinson)). The stained samples were washed and fixed in 1% paraformaldehyde fixative. The samples were stored at 4°C in the dark until fluorescence activated cell sorter (FACS) analysis.

Cells were analyzed by using a FACScan flow cytometer (Becton Dickinson, CA, USA) and CellQuest analysis program (Becton Dickinson). Forward light scatter, side light scatter, FL1 (FITC), FL2 (PE) and FL3 (CY-Chrome™) were used. A total of 25,000 ungated events were collected from lung samples, and 10,000 events from spleen and lymph node samples. The proportions of both $CD3^+$ and $CD4^+$ cells within the $CD45^+$ population in the lymphocyte gate were measured. Total activated and non-activated T cells, and cell numbers of other populations were calculated by multiplying the calculated total cell number in each organ (lung, spleen and lymph nodes) with the proportion of the corresponding cell population from $CD45^+$ cells.

4.6.4 LDH, total protein, albumin and hemoglobin analyses (I - V)

Lactate dehydrogenase (LDH) concentration in BALF was analyzed by Cytotoxicity Detection Kit (Boehringer Mannheim, GmbH, Germany) with minor modifications. The absorbances were measured by ELISA reader at the wavelength of 492 nm.

Total protein concentration in BALF was determined by the modified Lowry method, DC Protein Assay (Bio-Rad, Hercules, CA, USA). The absorbances were measured by ELISA reader at the wavelength of 690 nm.

The albumin concentration in BALF was at first analyzed by the modified Doumas colorimetric method (Procedure No. 631, Sigma, USA) (I). The absorbances were measured at the wavelength of 628 nm (Philips PU 8750, Great Britain or Shimadzu UV-1201, USA). To improve the specificity and sensitivity, albumin levels were later analyzed by the ELISA-method (II - V). Antibody pairs were from Bethyl Laboratories (Montgomery, TX, USA) and analyses were done according to the manufacturer's instructions. Briefly, 96-well microtiter plates were coated with monoclonal capture antibody, and the albumin in the samples and standards were allowed to adhere. BALF samples (1:900) and the standards were diluted in Diluent. HRP-conjugated second antibody was used. TMB solution was used as the substrate. The absorbances were measured by ELISA reader at the wavelength of 450 nm.

Hemoglobin concentrations in the supernatants of hemolyzed cell pellets were analyzed by using the modified colorimetric Stadie method (Procedure No. 525, Sigma, USA). Shortly, when samples mixed with Drabkin's reagent, and methemoglobin standards were incubated in 96-well plate, the absorbances were measured by ELISA reader at the wavelength of 540 nm. Hemoglobin concentrations were expressed as μg/ml BALF.

4.6.5 Histopathological analysis (I - V)

Tissue samples from lungs, lymph nodes, spleen and liver of non-lavaged mice, stored in 10% buffered formalin were trimmed, dehydrated, embedded in paraffin, cut into 5 μm sections and stained with hematoxylin and eosin. Histopathological changes were evaluated with a light microscope.

In the *M. terrae* study (II), to evaluate the mycobacterial infiltration especially in the lungs, sections including the left lung and the spleen from three animals per each group in the time-course experiment were stained for mycobacteria with standard Ziehl-Neelsen or Auramine-Rhodamine. Infiltration was subjectively graded as none, scattered, moderate, strong and very strong from Ziehl-Neelsen stained sections under the light microscope. Identification of the mycobacteria was confirmed from Auramine-Rhodamine stained sections under fluorescence microscopy.

4.6.6 Genotoxicity (V)

Genotoxicity was analyzed by using the alkaline Single Cell Gel (SCG) Assay to measure DNA damage in blood leukocytes. Determination of DNA damage with the SCG assay was done according to Singh *et al.* (1988) with some modifications. Briefly, a whole-blood sample was spread in low-melting agarose on a microscope slide covered with normal-melting agarose. The cells were lysed, and electrophoresis was performed. After neutralization, the slides were stained with ethidium bromide and analyzed by using an automated image analysis system (Komet 4.0.2., Kinetic Imaging Ltd, UK). The comet response parameters used in the statistical analysis of the data were tail DNA (tail%DNA), tail extent moment (tail length × tail%DNA/100), Olive tail moment [(tail mean - head mean) × tail%DNA/100] and tail length.

4.6.7 Statistical analysis (I - V)

The normally distributed data, with equal variances between the groups, were assessed using analysis of variance (ANOVA) and Dunnett's test: exposed groups were compared to carrier control group. In case the variances were unequal, ANOVA and Dunnett's C tests were used. Otherwise Kruskall-Wallis and Dunn's tests were performed. The difference was considered significant at $p < 0.05$.

5 RESULTS

5.1 Cytokine responses (I - V)

All the tested microbes enhanced TNFα and IL-6 production into bronchoalveolar lavage fluid (BALF) after a single microbial dose. However, there were marked differences in the time-courses of the responses, and in potency of the microbes to provoke the cytokine production.

TNFα in BALF (I - IV)

At equivalent volumetric doses, the most intense rapid TNFα response was induced by *S. californicus* (Fig. 1A). The cytokine level was more than 13 times higher in the *S. californicus* group than in the control group at 6 hours after the instillation. *M. terrae* provoked a rather similar acute response, the mean concentration being 83% of the response caused by *S. californicus*. The TNFα response induced by *M. terrae* was biphasic, including an intense acute phase and a sustained phase that lasted over 14 days. The major elevations in TNFα levels provoked by the other microbes had disappeared within 3 days. The spores of *A. versicolor* caused a rapid TNFα response, which appeared later than the responses induced by the other microbes. The response of *A. versicolor* peaked at 24 hours, and at the equal volumetric dose (5×10^6 spores), the response was almost 7-fold compared to the controls. *P. spinulosum* exposure provoked an over 8-fold increase in the TNFα level at 6 hours, but the response disappeared faster than the responses caused by the other microbes.

The 24 h time point in dose-response experiments was a compromise for several of the parameters, and was not optimal for comparison of microbe-induced TNFα responses. However, it revealed that already the dose of 1×10^6 spores of *A. versicolor* induced TNFα production (Fig. 1B). Both fungal species caused a very strong TNFα response at the highest dose level (*A. versicolor* 1×10^8 spores; *P. spinulosum* 5×10^7 spores), but the volumetric doses were large due to greater particle size of the spores compared to the bacteria, and it is possible that the volume exceeded the limit of overloading (see Discussion 6.1).

In consideration of both the dose-responses and the time-courses, the potency order and the lowest particle doses to cause TNFα responses were: *S. californicus* (1×10^8 spores) and *M. terrae* (1×10^8 cells) with approximately equal potency. The highest doses of *A. versicolor* and *P. spinulosum* were excluded from the evaluation, because they were in the range of the overloading dose (see 6.1). *S. californicus* caused severe inflammation already at the lower exposure level, whereas *M. terrae* provoked a sustained response. *A. versicolor* (1×10^6 spores) had approximately an equal potency with *P. spinulosum* (5×10^6 spores) to evoke the TNFα response. At equal volumetric doses, the fungal species had a lower potency to induce the TNFα response than the bacteria.

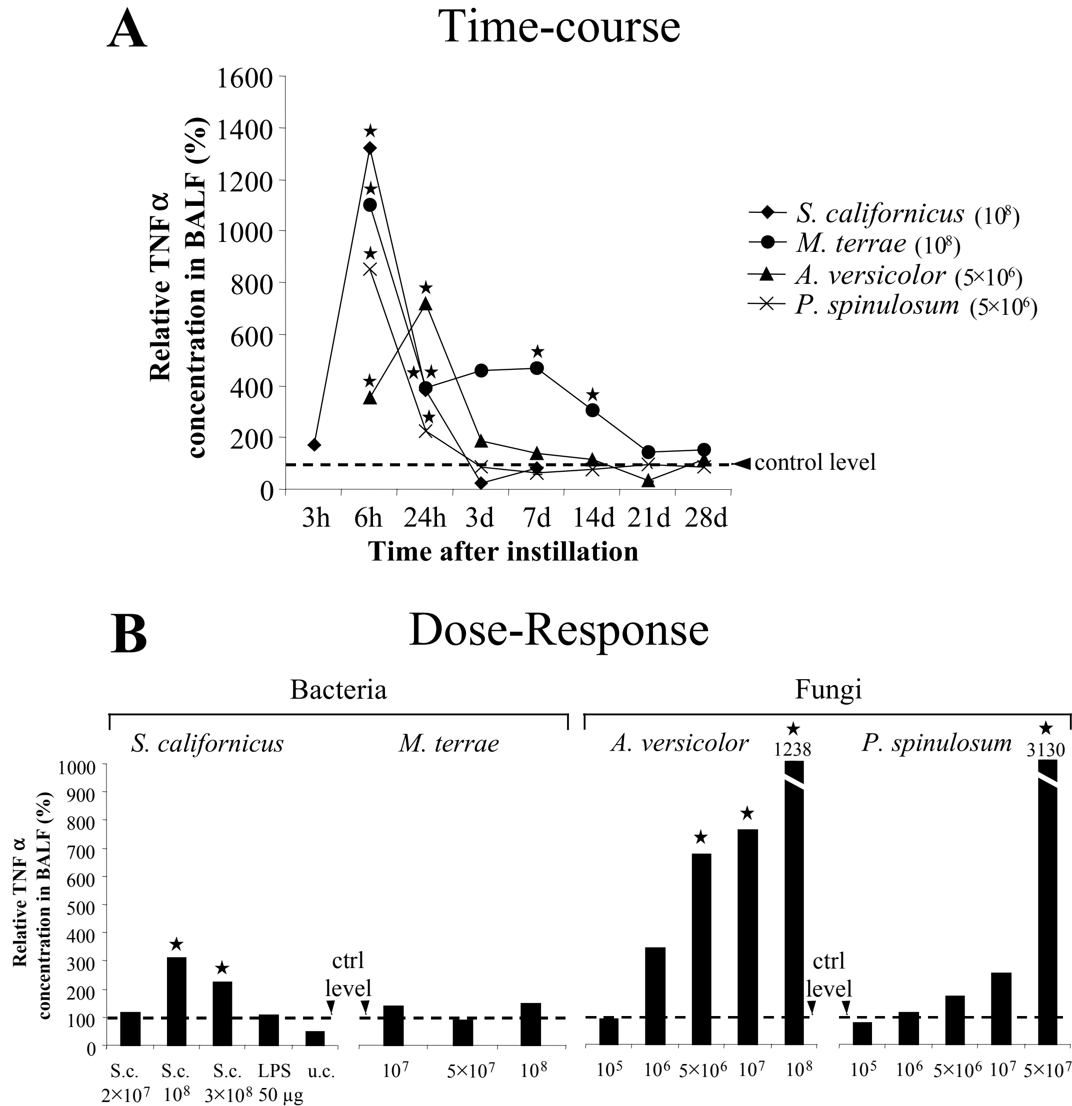

Figure 1. Time-course (A) and dose-response (at 24 hours) (B) of the relative TNFα concentrations in bronchoalveolar lavage fluid (BALF) after the instillation of a single dose of microbial spores or cells. The mean concentrations of the carrier control groups have been set to 100% (ctrl level). S.c. = *S. californicus*; LPS = lipopolysaccharide; u.c. = untreated control. Asterisk indicates a statistically significant difference from the control group in the original data ($p < 0.05$). (I - IV)

IL-6 in BALF (I - IV)

S. californicus clearly induced the highest IL-6 levels in BALF at equal volumetric doses (Fig. 2A). This response peaked later than the TNFα response by the same microbe. At 24 hours after the dosing, the IL-6 level was over 20 times higher than that seen in the control group. The other microbes were less potent, and their IL-6 responses peaked earlier, already at 6 hours (Fig. 2A). *A. versicolor* exposure caused the next strongest effects (Fig. 2A and 2B). The IL-6 response was massive at the dose level 1×10^8 spores of *A. versicolor*. *P. spinulosum* and *M. terrae* caused the weakest IL-6 responses, but the response provoked by *P. spinulosum* had subdued already by 24 hours (Fig. 2A and 2B).

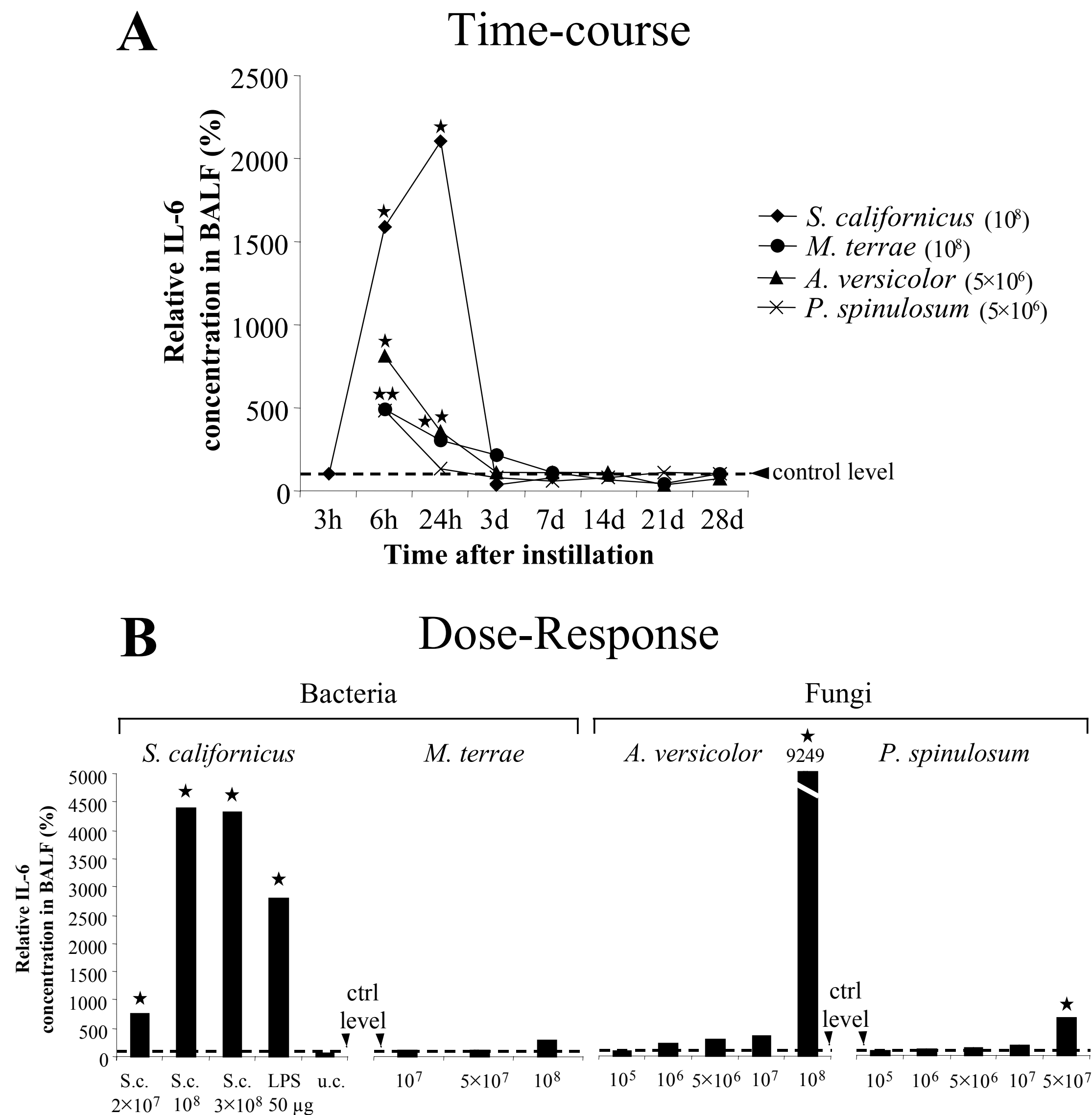

Figure 2. Time-course (A) and dose-response (at 24 hours) (B) of the relative IL-6 concentrations in bronchoalveolar lavage fluid (BALF) after the instillation of a single dose of microbial spores or cells. The mean concentrations of the carrier control groups have been set to 100% (ctrl level). S.c. = *S. californicus*; LPS = lipopolysaccharide; u.c. = untreated control. Asterisk indicates a statistically significant difference from the control group in the original data ($p < 0.05$). (I - IV)

Based on the similar assessment as for TNFα, the rank order of the microbial potential to provoke IL-6 response in the lungs was: *S. californicus* (2×10^7 spores), *A. versicolor* (1×10^6 spores), and then *M. terrae* (1×10^8 cells) and *P. spinulosum* (5×10^6 spores) with an approximately equal potency.

TNFα and IL-6 in BALF after repeated dosing (V)

After repeated dosing of the spores of *S. californicus* (2×10^3, 2×10^5, and 2×10^7 spores) no changes were observed in TNFα or IL-6 levels in BALF at 24 hours after the last (6th) weekly dosage.

TNFα and IL-6 in serum (I - V)

Serum TNFα and IL-6 levels were analyzed to study whether microbial exposure in the lungs resulted in a systemic cytokine response. Cytokine concentrations in serum were markedly lower than in BALF. The levels were frequently below the detection limits of the assays. A single dose of *S. californicus* (1×10^8 and 3×10^8 spores) increased slightly IL-6 levels at 24 hours (I). Both cytokines peaked at 6 after the instillation of 1×10^8 spores, concomitantly with the elevated concentrations in lungs. The cytokine responses were not detectable at lower microbial dose, even after repeated dosing of *S. californicus* (V). From the other microbes, only *A. versicolor* affected the cytokine levels in serum (III). The highest dose (1×10^8 spores) increased the IL-6 level at least 24-fold as compared to controls at 24 hours after the dosing, whereas smaller doses of this microbe did not affect the cytokine levels. LPS (50 µg), a positive control, increased serum cytokine levels, especially that of IL-6.

5.2 Expression of inducible nitric oxide synthase (I - V)

Inducible NOS was expressed in BAL cells after *S. californicus* and *M. terrae* exposure, whereas exposure to *A. versicolor* or *P. spinulosum* did not provoke any detectable iNOS expression in lavaged cells. The time-course of the iNOS response was very different between the bacterial strains. Inducible NOS protein was detectable at 24 hours after *S. californicus* exposure (I), whereas exposure to *M. terrae* did not cause the response before day 7, and the protein was detectable up to 28 days (II).

Even though the lowest dose of *S. californicus* (2×10^7 spores) induced iNOS expression after a single dose (I), surprisingly the expression was not detectable after repeating the same dose (V).

5.3 Inflammatory cell responses (I - V)

The microbes increased the total number of inflammatory cells in BALF though their potency and the time-course were different. At the equal volumetric doses, *S. californicus* provoked the highest increase of inflammatory cells (Fig. 3A and 3B). In addition, already the lowest dose (2×10^7 spores) caused the maximum, i.e. over 6-fold increase in the total cell number in BALF (Fig. 3B). Neutrophils were the most typical cells for the acute inflammatory cell response at 24 hours, macrophages peaked at 3 days, and lymphocytes at 7 days (I). The lowest dose (2×10^7 spores) caused the highest lymphocyte numbers, at 24 hours. LPS recruited neutrophils but not lymphocytes or macrophages into the airways at 24 hours.

When compared at equal volumetric doses *M. terrae* provoked the second strongest response, which was biphasic (Fig. 3A). The acute inflammatory cell response peaked at 24 hours, the cell number remained elevated thereafter, and the second phase appeared from 7 days (Fig. 3A). The strength of the acute response by *M. terrae* was about one-third of that caused by *S. californicus*. The cell numbers were still increased even on day 28, contrary to the responses provoked by the other microbes. In the acute phase, these excess inflammatory cells were mainly neutrophils, whereas macrophages and lymphocytes were the predominant cells in the later phase (II). The mycobacterium exposure provoked also an exceptionally long neutrophil response, which lasted for up to 21 days.

A. versicolor caused recruitment of inflammatory cells into the airways dose-dependently beginning at the dose 1×10^6 spores. At that dose, the total cell number was 32% above the control level (Fig. 3B). When equal volumetric doses were compared, the *A. versicolor* induced cell response was 34% of that caused by *S. californicus* (Fig. 3A). The highest dose (1×10^8) caused a drastic inflammatory cell flow into the airways. Again, neutrophils were responsible for the acute increase of inflammatory cells at 24 hours, macrophages peaked at 3 days, and lymphocytes at 7 days (III). Thus, the pattern resembled that induced by a single dose exposure of *S. californicus* (I). However, none of the *A. versicolor* doses increased lymphocytes at 24 hours (III). *P. spinulosum* caused only minor inflammatory cell responses even at the highest dose level (Fig. 3A and 3B). The increase in inflammatory cells was mainly due to neutrophils (IV).

None of the exposures recruited eosinophils into the airways after a single dose.

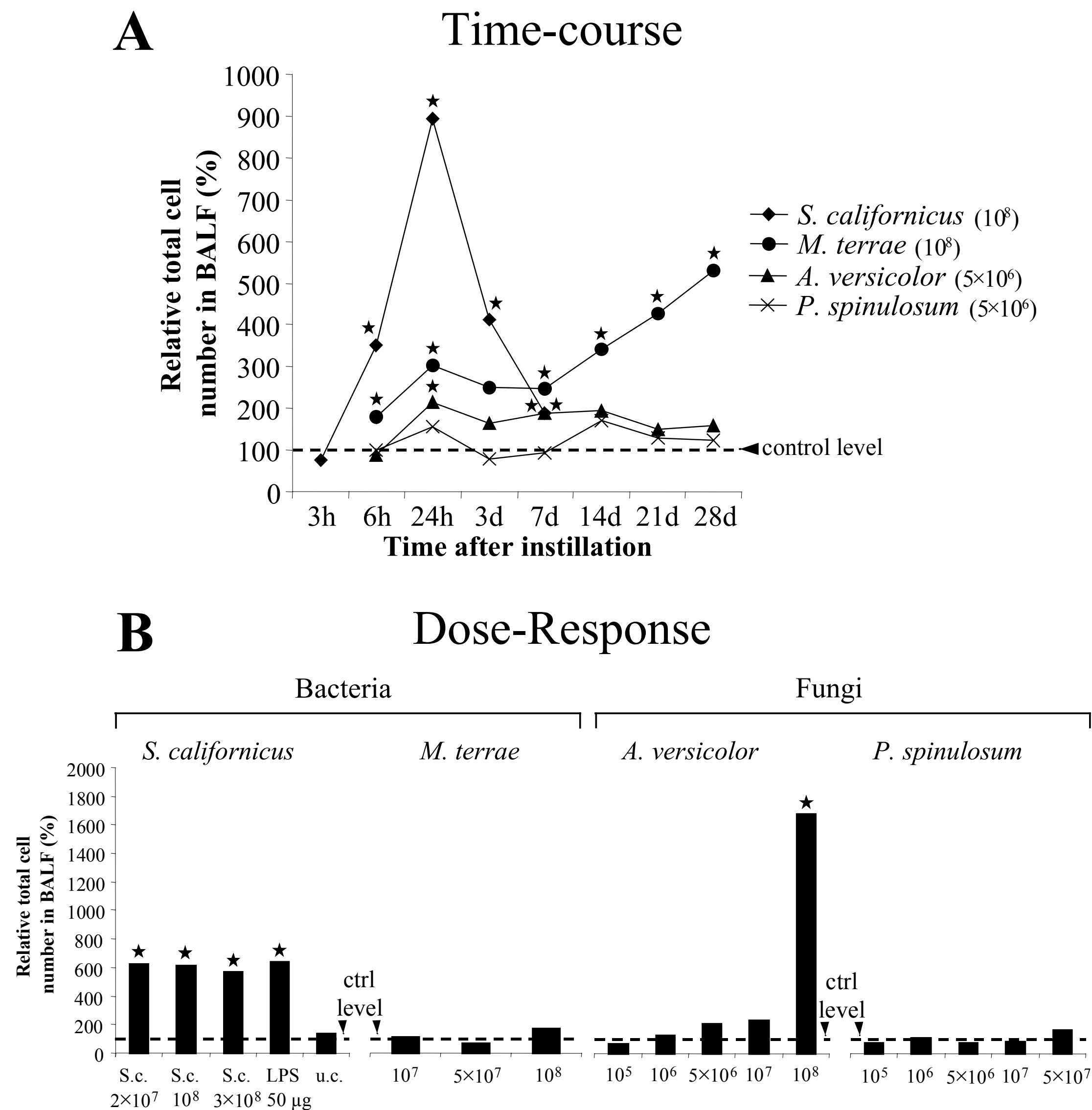

Figure 3. Time-course (A) and dose-response (at 24 hours) (B) of the relative total cell numbers in bronchoalveolar lavage fluid (BALF) after the instillation of a single dose of microbial spores or cells. The mean concentrations of the carrier control groups have been set to 100% (ctrl level). S.c. = *S. californicus*; LPS = lipopolysaccharide; u.c. = untreated control. Asterisk indicates a statistically significant difference from the control group in the original data ($p < 0.05$). (I - IV)

The potency order and the lowest particle doses to cause inflammatory cell responses were: *S. californicus* (2×10^7 spores) and *M. terrae* (1×10^8 cells) with an approximately equal potency. *S. californicus* provoked a very strong acute response but *M. terrae* provoked also the sustained response. *A. versicolor* (1×10^6 spores) exposure recruited inflammatory cells into the airways more efficiently than *P. spinulosum* (5×10^6 spores) exposure. Based on volumetric doses, the fungal species had lower potency to evoke an inflammatory cell response than the bacteria.

Repeated dosing of 2×10^7 spores of *S. californicus* caused a major recruitment of inflammatory cells into the airways, the total cell number was over 18-fold compared with the control (V). The inflammatory cell response consisted mainly of neutrophils and macrophages. Moreover, the mean lymphocyte number was 9-fold in the spore-exposed group. The dose of 2×10^5 spores increased the mean total cell number by 29 %. This was mainly due to the increased numbers of macrophages. Eosinophils were only occasionally observed in BALF. The lowest dose (2×10^3 spores) did not increase the numbers of inflammatory cells in BALF.

Red blood cells in BALF may indicate severe increase in vascular permeability and/or vascular damage. Single doses of *S. californicus* (2×10^7, 1×10^8 and 3×10^8 spores) increased equally well the hemoglobin concentration in BALF at 24 hours (I). The effect was transient, being detectable already at 6 hours but had disappeared by 3 days. Also *A. versicolor* provoked red blood cell leakage into airways, but the effect was detectable only at the highest dose (1×10^8 spores) at 24 hours (III). LPS (50 µg) elevated the hemoglobin concentration at 24 hours (I). Neither repeated dosing of the spores of *S. californicus* nor single dosing of the other microbial species caused any marked increases in red blood cell leakage into the airways (II, IV and V).

5.4 Albumin, total protein and LDH (I - V)

Albumin in BALF may indicate increased vascular leakage due to inflammation or vascular damage. The strongest response was provoked by *M. terrae*: albumin in BALF was on the increase from 24 hours and peaked at 14 days after bacterial exposure (Fig. 4A). *S. californicus* caused the most rapid albumin response in the airways, in 6 hours and it lasted over 24 hours (Fig. 4A). *A. versicolor* induced a slower response than the bacterial species, and its intensity was similar to that evoked by *S. californicus*, but it lasted for a longer time. *P. spinulosum* exposure caused the mildest changes in the albumin level in BALF (Fig. 4A and 4B).

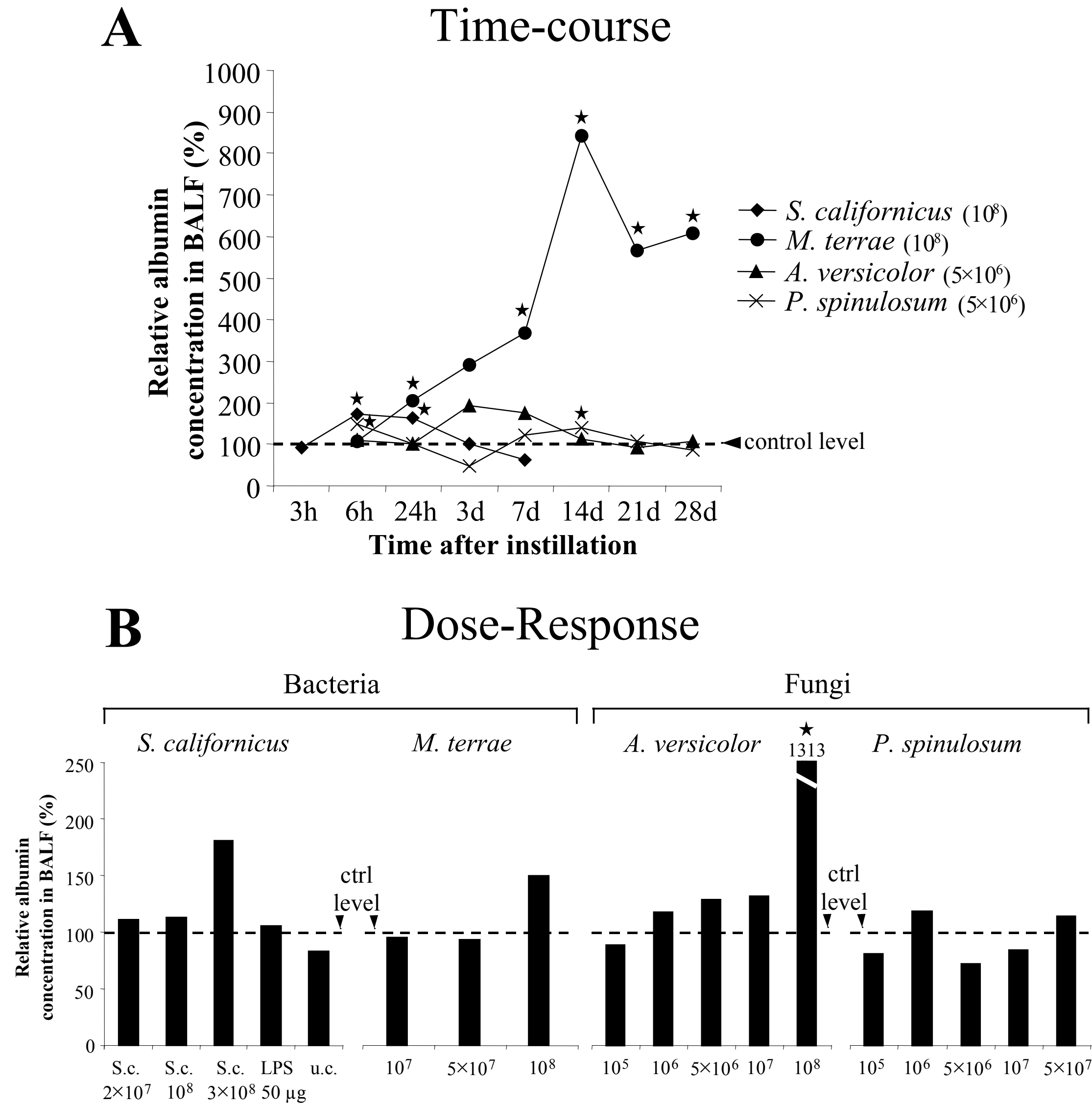

Figure 4. Time-course (A) and dose-response (at 24 hours) (B) of the relative albumin concentration in bronchoalveolar lavage fluid (BALF) after the instillation of a single dose of microbial spores or cells. The mean concentrations of the carrier control groups have been set to 100% (ctrl level). S.c. = *S. californicus*; LPS = lipopolysaccharide; u.c. = untreated control. Asterisk indicates a statistically significant difference from the control group in the original data ($p < 0.05$). (I - IV)

The potency order and the lowest particle doses to cause the observed albumin responses were: *M. terrae* (1×10^8 cells), then *S. californicus* (2×10^7 spores) and *A. versicolor* (1×10^6 spores) with approximately equal potency, and then *P. spinulosum* (5×10^6 spores).

The dose of 2×10^7 spores of *S. californicus* caused a significant albumin leakage into the airways after repeated dosing (V).

The increased total protein in BALF is indicative of tissue/cell damage and/or vascular leakage. The total protein levels resembled the albumin levels in BALF suggesting that the majority of the protein was albumin, which had leaked from pulmonary capillaries (Fig. 5A and 5B). Again, the mycobacterium exposure provoked a sustained response, and the highest levels were observed as late as on 14 and 21 days. *S. californicus* and *A. versicolor* caused transient responses. *P. spinulosum* exposure had only minor effects on the total protein level in BALF.

Based on similar assessment, the potency order and the lowest particle doses to cause observed increase in total protein level in BALF were: *M. terrae* (1×10^8 cells), then *S. californicus* (2×10^7 spores) and *A. versicolor* (1×10^5 spores) with approximately equal potency, and then *P. spinulosum* (5×10^7 spores).

The 2×10^7 spores of *S. californicus* provoked a similar increase in the total protein level in BALF after repeated dosing (V) as 1×10^8 spores after a single dose (I). The lower doses did not affect total protein levels in BALF after repeated dosing.

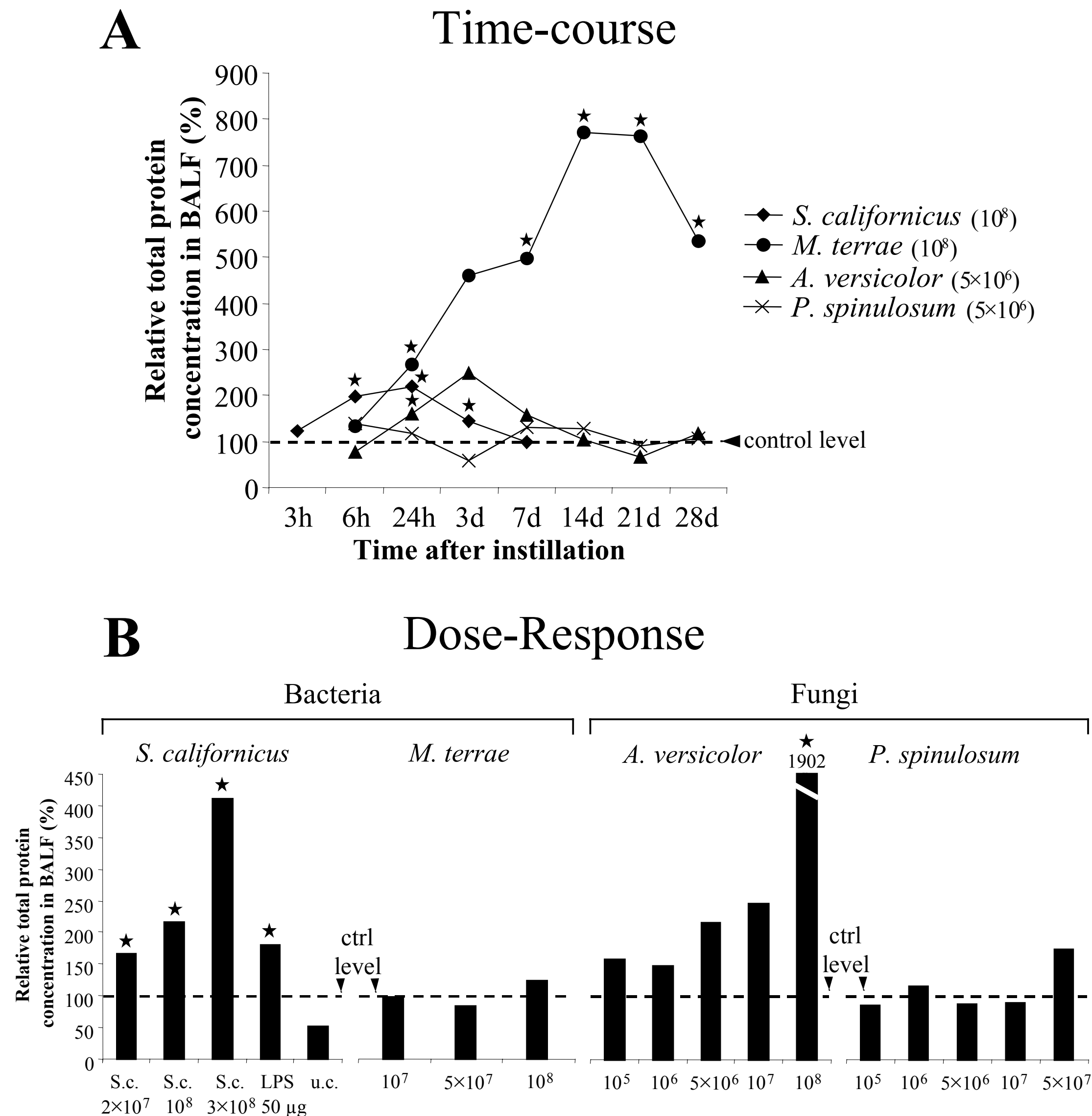

Figure 5. Time-course (A) and dose-response (at 24 hours) (B) of the relative total protein concentrations in bronchoalveolar lavage fluid (BALF) after the instillation of a single dose of microbial spores or cells. The mean concentrations of the carrier control groups have been set to 100% (ctrl level). S.c. = *S. californicus*; LPS = lipopolysaccharide; u.c. = untreated control. Asterisk indicates a statistically significant difference from the control group in the original data ($p < 0.05$). (I - IV)

LDH was measured as an indicator of increased cell death in lungs. Acute increases of LDH in BALF were observed during *S. californicus*, *M. terrae* and *A. versicolor* exposure (Fig. 6A and 6B). *M. terrae* provoked the strongest and also a sustained response, which lasted to the end of the experiment. *P. spinulosum* did not cause any marked cytotoxicity at any dose or time point studied.

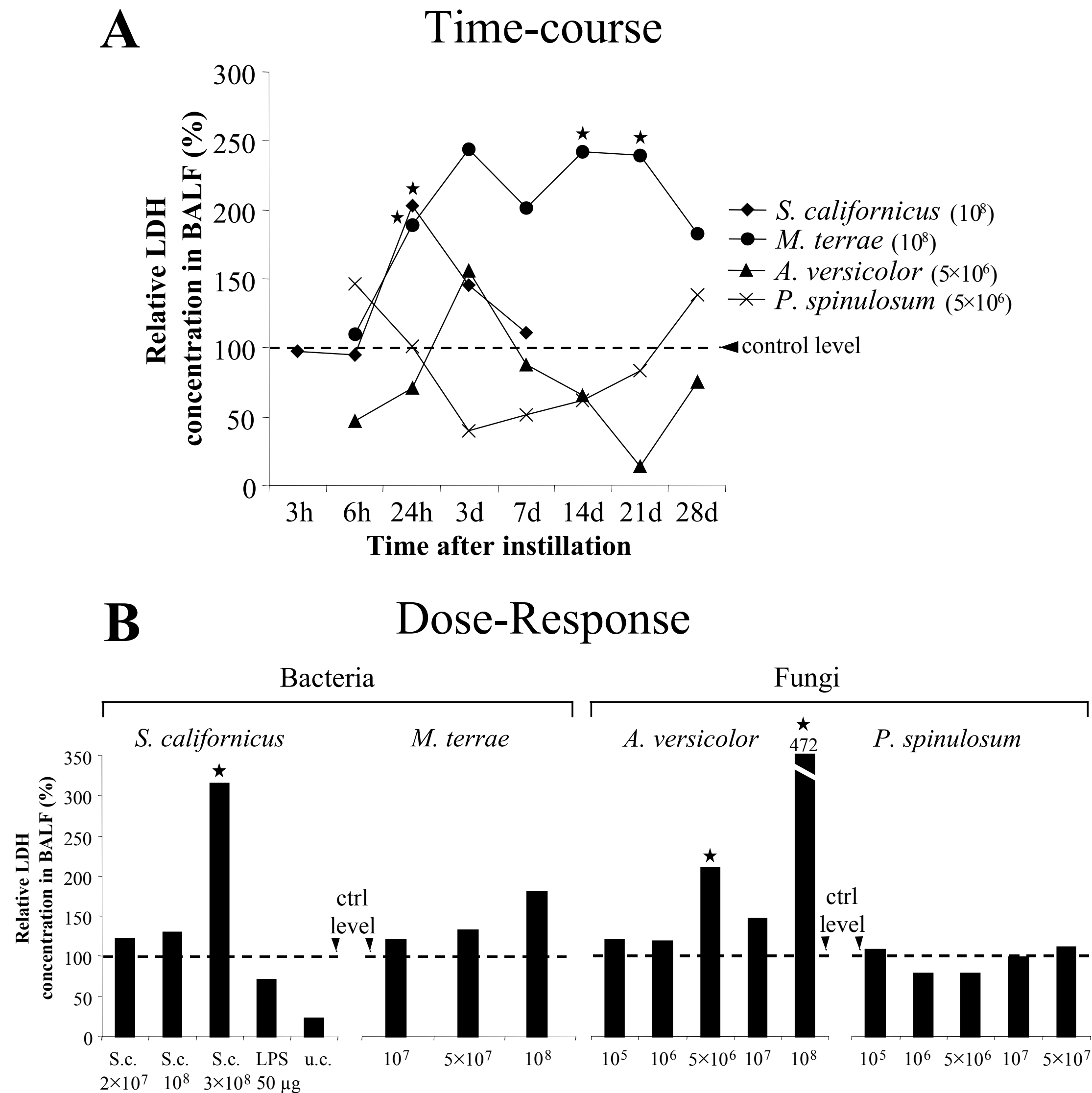

Figure 6. Time-course (A) and dose-response (at 24 hours) (B) of the relative lactate dehydrogenase (LDH) concentration in bronchoalveolar lavage fluid (BALF) after the instillation of a single dose of microbial spores or cells. The mean concentrations of the carrier control groups have been set to 100% (ctrl level). S.c. = *S. californicus*; LPS = lipopolysaccharide; u.c. = untreated control. Asterisk indicates a statistically significant difference from the control group in the original data ($p < 0.05$). (I - IV)

The potency order in the LDH responses was: *M. terrae* (1×10^8 cells), *S. californicus* (1×10^8 spores), *A. versicolor* (5×10^6 spores), and *P. spinulosum* (not toxic).

The LDH level in the group exposed repeatedly to *S. californicus* (2×10^7 spores) was almost 2.5-fold compared to the controls, though the difference was not statistically significant (V). The response was greater than that observed after a single dose of the same or even a higher dose (1×10^8 spores) (I).

5.5 Histopathological changes (I - V)

Histopathological analysis confirmed the inflammatory changes in the lungs. The spores of *S. californicus* caused a strong acute dose-dependent inflammation in lungs after a single dosage (I). Already 2×10^7 spores caused mild or mild/moderate inflammation at 24 hours. When the dose was increased to 1×10^8 spores, the inflammation was mild/moderate already after 6 hours from the instillation, most severe at 24 hours, but mild inflammatory changes were still seen after 3 days. The histopathological signs of inflammation had elapsed by 7 days. The inflammation induced by the positive control LPS (50 µg) was mild/moderate after 24 hours. Also the carrier buffer caused some mild inflammatory changes.

Neutrophils were the predominant cells in the acute inflammatory changes in the lungs during *M. terrae* exposure (II). They increased dose-dependently at 24 hours. Macrophages and other mononuclear cells were increasingly present in alveolar and partly in the bronchiolar spaces on day 3, and their occurrence in alveoli was increased for up to 28 days after exposure to *M. terrae* (1×10^8 cells). Mononuclear cells, such as lymphocytes and macrophages were typically seen peribronchiolarly and perivascularly. This response seemed to be the most severe on days 14 and 21. Based on mycobacterial staining, mycobacteria were present in lungs at least till 28 days after a single bacterial dose. The highest levels were observed at 24 hours. Presumably all mycobacterial cells had not yet been phagocytized at 6 hours, and thus they might have been partly lost during the sample processing. The number of mycobacteria in the lungs was decreasing from 14 days after the dosing.

The inflammation-related histopathological changes in lungs by *A. versicolor* were dose-dependent at 24 hours (III). An increased number of neutrophils was observed above 1×10^5 spores. However, the histopathological changes were mild to moderate with doses lower than 1×10^8 spores. Neutrophils were the most prominent cells up to 24 hours, whereas mononuclear cells such as macrophages and lymphocytes were the predominant cells from 3 to 7 days after instillation of 5×10^6 spores. Moreover, few animals with granuloma like formations were detected at these two time points, and peribronchiolar and -vascular aggregates of lymphocytes were observed at 7 days. Thereafter, the changes were minor, and all exposure-induced changes had disappeared by 28 days.

P. spinulosum induced the mildest histopathological changes. However, dose-dependent neutrophilic inflammation in the lungs was observed with doses higher than 1×10^6 spores at 24 hours (IV). Exposure to 5×10^6 spores induced a neutrophil response that disappeared after 24 hours, and the number of mononuclear cells was slightly increased peribronchially and -vascularly between 3 and 14 days.

Repeated dosing of *S. californicus* (2×10^7 spores) induced a moderate to abundant increase in the numbers of mononuclear cells and neutrophils in the alveoli and in bronchiolar lumen. The amount of peribronchial and vascular mononuclear cells was also increased. Moreover, in one out of three mice, these mononuclear cells also created granuloma-like formations. Also the lowest dose (2×10^3 spores) caused some neutrophil responses, but the mid-dose (2×10^5 spores) did not cause any histopathological changes.

M. terrae exposure (1×10^8 cells) induced a reactive change, i.e. mild follicular hyperplasia in lymph nodes associated with the lungs from 14 to 28 days (II). The highest single dose of *S. californicus* (3×10^8 spores) cause mild inflammatory changes in mediastinal lymph nodes at 24 hours (I). Neither *A. versicolor* nor *P. spinulosum* caused any clear exposure related changes in lymph nodes (III and IV). Repeated dosing of 2×10^7 spores of *S. californicus* induced reactive changes in lymph nodes, whereas this effect was not observed at the lower doses of the microbe (2×10^3 and 2×10^5 spores) (V).

None of the exposures caused any clear exposure related histopathological changes in the spleen or the liver (I - V).

5.6 Lymphocyte populations in lungs, lymph nodes and spleen (V)

Repeated dosing of the spores of *S. californicus* affected lymphocyte populations in the lungs, lymph nodes and the spleen.

Lungs

The highest dose of *S. californicus* (2×10^7 spores) induced the strongest effects in the lungs. It increased the total cell number by more than five-fold, as well as elevated both the T cell ($CD3^+$ cells) and the activated T cell ($CD3^+CD25^+$) numbers. The number of T lymphocytes more than doubled and the activated T cells increased by 3.5-fold compared to the control. In addition, the proportion of activated T cells ($CD3^+CD25^+$ cells/$CD3^+$ cells) was clearly higher (9%) in the spore-exposed group than in the control group. The majority of the T cells were helper T cells ($CD3^+CD4^+$), even though also the $CD3^+CD4^-$ cell population including killer T cells ($CD3^+CD8^+$) and $\gamma\delta^+$ T cells almost doubled at the highest dose level. However, the greatest lymphocyte cell number was observed in the $CD3^-CD4^-$ population (includes B lymphocytes and natural killer cells), the increase in these cells being almost 5-fold. Also the amount of unconventional $CD3^-CD4^+$ lymphocytes increased at the highest dose level more than 7-fold. Although, the lower spore doses did not markedly affect the cell populations, the cell numbers tended to be lower at the spore dose of 2×10^5 compared to that of 2×10^3.

Lymph nodes

The strongest increase in the total cell number (41%) in lymph nodes was detected at the lowest repeated dose of *S. californicus* (2×10^3 spores). A similar dose-response pattern was seen in all cell populations. Even though the T cell number increased during the spore exposure, the proportion of activated T cells remained low. The most notable change in the cell populations at the lowest spore dose was a 53% increase in the number of $CD3^+CD4^-$ cells. The greatest changes induced by the highest spore dose (2×10^7) were the increases in the total cell number (19%) and the number of activated lymphocytes (18%). The mid-dose (2×10^5) had only minor effects on the total cell number and cell populations.

Spleen

The total cell number in the spleen decreased during the repeated exposure to the spores of *S. californicus*. This effect was most obvious at the dose of 2×10^5 spores (38% decrease). The effect was partly reversed by the highest spore dose (2×10^7), when the total cell number was only 13% below the controls. The numbers of T cells, activated T cells, helper T cells,

and especially the amount of other T cells ($CD3^{+}CD4^{-}$) was decreased by all spore doses. The strongest effect was again detected at the mid-dose (2×10^{5} spores), when the decrease varied between 36% and 52%. The dose of 2×10^{3} spores decreased the numbers of $CD3^{+}$ to a greater extent than it depressed $CD3^{-}$ cell numbers. The higher doses, especially the mid-dose (2×10^{5} spores) decreased both $CD3^{-}CD4^{-}$ and $CD3^{-}CD4^{+}$ numbers. Although the changes in the cell populations in lymph nodes and spleen were not statistically significant, the trends were clear.

5.7 Genotoxicity (V)

DNA damage assessed by the comet response was not detected in blood leukocytes after repeated dosing of the spores of *S. californicus* into the airways.

6 DISCUSSION

There is convincing epidemiological evidence indicating that moisture-damage in buildings can provoke adverse health effects. Previous *in vitro* experiments have also shown that bacterial and fungal species isolated from moisture-damaged buildings can cause inflammatory and toxic responses (Hirvonen et al., 1997b; Huttunen et al., 2000; Huttunen et al., in press). However, the data concerning *in vivo* effects after experimental exposure in lungs have largely been missing. There has not been any comparative knowledge on the potential of different moisture indicator microbes to cause adverse effects. In addition, it has not been known how well the *in vitro* experiments can predict *in vivo* effects. This thesis has addressed these particular points.

This is the first time when the inflammatory and toxic potential of the microbes isolated from moisture-damaged buildings has been compared in a systematic way after airway exposure. Thus, a mouse model was devised. The responses provoked by *S. californicus* and *M. terrae* have not been previously investigated *in vivo* at all. Moreover, this is one of the first studies when systemic effects and the effects after repeated airway exposure of a microbe from moisture-damaged building have been investigated.

6.1 Evaluation of the mouse model (I - V)

Airways are presumably the most important route of exposure in occupants of moisture-damaged buildings. The route of exposure is critical for toxicological evaluation, since for example, mycotoxins may be more toxic after airway exposure than after per oral dosing (Creasia et al., 1990). The selected exposure method is also important. In intratracheal instillation, the dose is accurate. The method has been evaluated to be sufficiently similar to inhalation exposure, since it has been routinely used in studies on the pneumotoxic potential of respirable particles (Reasor and Antonini, 2001), even though the particle deposition and distribution patterns are slightly different from that of the inhalation method (Brain et al., 1976; Pritchard et al., 1985). Other advantages of the mouse model are that the immunological system of the mouse is relatively well known, and *in vitro* findings can be compared to *in vivo* observations in the same species.

Both the dose-response and time-course experiments were considered necessary for the evaluation of the inflammatory and toxic potential of the microbes. The time-course experiment indicates the peak and duration of response, and the dose-response experiment reveals the lowest active dose. However, to reduce the size of the experiments, the time points for sampling had to be kept reasonable. The time point of 24 hours for the dose-responses was a compromise between different responses, and therefore, was not optimal for all of the parameters studied. The acute inflammatory cell responses peaked at 24 hours, whereas the cytokine responses generally peaked earlier. Although the cytokine responses were still detectable at that time, the ultimate lowest effective doses could not be observed.

The dose needs special attention, because microbes are different in their size. The spores are also vehicles for microbial toxins (Sorenson, 1999) and other active agents which are either inside the spores or on the cell walls. It has been shown that both viable and nonviable spores may initiate inflammatory and toxic responses (Hirvonen et al., 1997c; Cooley et al., 2000). Furthermore, the pure particle effect may contribute to the response observed, and this has to be taken into consideration when the data are interpreted. Thus, it is relevant to use whole spores or intact microbes as an exposure. In addition, high doses of particles are known to cause the overloading phenomenon, i.e. non-specific effects due to physical overloading and the subsequent excess activation of the macrophages (Morrow, 1992). Responses induced by such high doses are toxicologically irrelevant and should be distinguished from the other exposure-induced effects. The dose to cause overloading in rat has been approximated to be 60 μm^3/alveolar macrophage, by estimating that there are 5×10^7 activated alveolar macrophages in rat lungs (Morrow, 1992). The overloading dose for mice is not known but it was approximated to be the same, and the number of activated alveolar macrophages in mouse lungs was assumed to be 1/10 of the amount in the rat (5×10^6 cells) (I - IV).

In this summary, to avoid the contribution of the overloading phenomenon to the comparison of the microbes, the volumetric dose (the total volume of the particles in the dose) was used as one criterion of the dose in addition to the particle number. The comparison was made at doses, which are below the overloading dose. Particle size determines the volumetric dose. In addition to the volume, the particle size determines conceivable amount of biologically active components inside the spores or microbial cells. Moreover, the surface area of the dose, i.e. the conceivable amount of biologically active cell wall structures, is determined by the size of the particle. As seen in Figure 7, the volumetric doses and surface areas of the microbial doses

with equal particle numbers may differ markedly. In order to produce truly representative dose-response curves, also high doses had to be included in the experiments. The calculations indicated that only the dose of 10^8 spores of *A. versicolor* (III) and 5×10^7 spores of *P. spinulosum* (IV) exceeded the approximated overload dose. This was, indeed, supported by biochemical responses, which increased abruptly at these doses. Moreover, the number of alveolar macrophages decreased, presumably because of overloading induced non-specific cytotoxicity. As stated, the effects induced by the highest doses of fungal spores were excluded from the final evaluation of the results (Table 7).

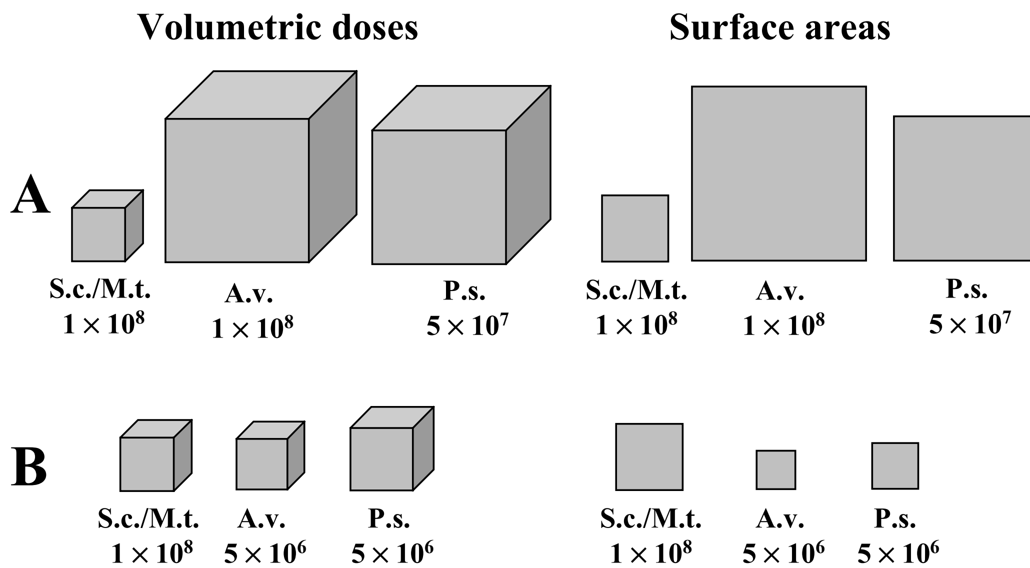

Figure 7. A) Relative volumetric doses and surface areas of the indicated microbial doses. B) Approximately equal volumetric doses and their surface areas. Particles were suggested to be spherical, and physical diameters of the spores of *S. californicus* (S.c.) and *M. terrae* (M.t.) cells 1 µm, and spores of *A. versicolor* (A.v.) 2.75 µm and *P. spinulosum* (P.s.) 3.25 µm.

The overall comparison of the microbes is difficult because both the volumetric dose and the number of the microbial particles must be taken into consideration. Therefore, the time-course studies were conducted with equal volumetric doses, and the dose-response studies over a wide particle number range, and this summary is a synthesis of the data. In the determination of the effective dose, the whole dose-response data were considered, not only the statistically significant changes.

6.2 Inflammatory and toxic potential of the microbes after single dosing (I - IV)

All the microbial strains provoked inflammatory changes in the lungs as assessed by biochemical and cell profile analyses in BALF and these responses were confirmed by

histopathological analysis. However, the microbes had a different potential to cause inflammatory and toxic responses, and both the magnitude and the time frame of the responses were different. Moreover, the duration of the responses clearly differed. Due to many differences in both microbial particles and induced responses, it is difficult to compare and arrange the microbes in a rank order according to their inflammatory and toxic potency, even though this would be useful in risk assessment. Comparison of bacterial and fungal species separately is easier since both particle numbers and volumetric doses are approximately equal. However, comparison of the bacteria and fungi was also necessary.

S. californicus provoked a very strong acute inflammatory response, even at the lowest dose level (2×10^7) (Table 7). The exposure induced strong and rapid TNFα, IL-6 and iNOS production, albumin leakage and heavy acute neutrophil infiltration into the airways. Later increases of macrophages and lymphocytes were also observed in the lungs typically for acute inflammation. *S. californicus* was the only microbe, which notably increased red blood cell flow into the airways. It was not possible to determine whether the spores were toxic to capillary endothelium or the acute inflammatory response was strong enough to increase red blood cell flow through the walls of the dilated capillaries. The effect of a single dose had decayed nearly within a week. The lowest effective single dose level remained unknown since the lowest spore dose tested resulted in a marked inflammatory response. Based on these acute responses, *S. californicus* can be considered as the most potent of these microbes (Table 7).

Table 7. Summary of the observed effects and effective doses of the microbes after a single dose.

Response	*S. californicus* (spores)	*M. terrae* (cells)	*A. versicolor* (spores)	*P. spinulosum* (spores)
TNFα in BALF	**10^8**[a] (2×10^7)[b] 1.[c]	**10^8 S** (5×10^7) 1.	**10^6** (10^5) 3.	**5×10^6** (10^6) 4.
IL-6 in BALF	**2×10^7** (N[d]) 1.	**10^8** (5×10^7) 3.	**10^6** (10^5) 2.	**5×10^6** (10^6) 3.
Inducible NOS in lavaged cells	**2×10^7** (N) 1.	**10^8 D** (5×10^7) 1.	**No effect** 3.	**No effect** 3.
Inflammatory cells in BALF	**2×10^7** (N) 1.	**10^8 S** (5×10^7) 1.	**10^6** (10^5) 3.	**5×10^6**[e] (10^6) 4.
Albumin in BALF	**2×10^7** (N) 2.	**10^8 S** (5×10^7) 1.	**10^6** (10^5) 2.	**5×10^6** (10^6) 4.
Total protein in BALF	**2×10^7** (N) 2.	**10^8 S** (5×10^7) 1.	**10^5** (N) 2.	**No effect** 4.
LDH in BALF	**10^8** (N) 2.	**10^8 S** (5×10^7) 1.	**5×10^6** (N) 3.	**No effect** 4.
Red blood cells in BALF	**2×10^7** (N) 1.	**No effect** 2.	**No effect** 2.	**No effect** 2.
Histopathology of lungs	**2×10^7** (N) 1.	**10^8 S** (5×10^7) 1.	**10^6** (10^5) 3.	**5×10^6** (10^6) 4.
Histopathology of lymph nodes	**3×10^8**[f] (10^8) 1.	**10^8 D** (5×10^7) 1.	**No effect** 2.	**No effect** 2.
IL-6 in blood	**10^8** (2×10^7) 1.	**No effect** 2.	**No effect** 2.	**No effect** 2.
Equal volumetric dose	**10^8**	**10^8**	**5×10^6**	**5×10^6**
The lowest dose evaluated	**2×10^7**	**10^7**	**10^5**	**10^5**
Doses in the range of overloading[g]	–	–	**10^8**	**5×10^7**

D = Delayed response. S = Acute and sustained response.
[a] The lowest effective dose.
[b] No observed effect level (NOEL) in this study.
[c] Rank order of descending potency.
[d] The lowest effective dose was below the lowest administered dose.
[e] Only a minor neutrophil response.
[f] Only mediastinal lymph nodes were analyzed.
[g] Excluded from the evaluation of the potency.

The marked acute inflammatory response caused by *M. terrae* was manifested at a higher dose level (1×10^8) than by *S. californicus* (2×10^7), whereas the inflammatory response was sustained for a considerably longer time. A single dose of *M. terrae* caused a biphasic inflammation which lasted at least for 4 weeks, suggesting that even a short intermittent exposure may provoke prolonged adverse effects. This was presumably due to the better ability of mycobacteria to avoid innate host defense mechanisms than the other microbes. However, intracellular growth of the microbe could not be detected by mycobacterial staining. Both biochemical and inflammatory cell responses differed between the two phases of the inflammatory response. Production of TNFα seemed to be associated with IL-6 response only in the acute phase, and with NO production only in the later phase. TNFα and NO have been shown to contribute to neutrophil recruitment during *M. bovis bacillus Calmette-Guérin* (BCG) exposure in mice (Menezes-de-Lima-Június et al. 1997). Thus, these mediators may have a role in the sustained neutrophil response observed during *M. terrae* lung exposure. Moreover, NO has been suggested to be an important factor in the host response against mycobacteria (Denis, 1991; Flesch and Kaufmann, 1991; Chan et al., 1992). The role of IL-6 is not clear in mycobacterial infections (Kopf et al. 1994; VanHeyningen et al. 1997).

The inflammatory cell response was also biphasic during exposure to *M. terrae*. Neutrophils were the most typical cells in the acute phase, whereas macrophages and lymphocytes were characteristic of the later phase. T cell-mediated immunity has been shown to be essential in the host defense against mycobacterial diseases (Holland et al. 1996), which is presumably one reason for the strong lymphocyte and macrophage responses after *M. terrae* exposure. In line with the current finding, sustained biphasic inflammatory response in the mouse lungs induced by another non-tuberculous mycobacterium, *M. bovis* BCG has been described earlier (Menezes-de-Lima et al., 1997). Intratracheal instillation of 10^5 living cells of *M. bovis* BCG provoked a lymphocyte response in the lungs, which peaked at 4 weeks after the dosing (Fulton et al., 2000). In particular, the neutrophil response was slower after *M. bovis* BCG exposure than the response induced by *M. terrae*. The sustained neutrophil responses in these studies suggest that neutrophils play a role in controlling non-tuberculous mycobacterial infections in mice. *M. terrae* was not acutely very toxic *in vivo*. However, it caused significant cytotoxicity especially 2 - 3 weeks after the single dose. This was possibly due to inflammatory cell-mediated killing of the infected cells, i.e. the cytotoxicity was inflammation-mediated.

The lowest exposure level of *M. terrae* to cause sustained inflammatory response after a single dose or upon repeated dosing cannot be predicted. Some responses, including monocyte recruitment and albumin leakage into the airways, were more intense during the later phase of the inflammation (Table 7). Since dose-response data revealed only the acute responses, the duration of the inflammation after lower dose exposure is not known. However, the potential of the mycobacterium to cause sustained inflammation suggests that it is, indeed, a harmful indoor air microbe.

A. versicolor induced an acute inflammation in the mouse lungs. Previously *A. versicolor* exposure has been reported to cause inflammatory changes also in the rat lungs (Sumi et al., 1994). In that study, production of IL-1β and inflammatory cell recruitment into the lungs were observed. These findings together indicate that exposure to the spores of *A. versicolor* causes production of several proinflammatory cytokines, including TNFα, IL-1 and IL-6. In line with the current findings, TNFα and IL-6 have been shown to be important components of the host defense in *Aspergillus fumigatus* induced inflammation in the mouse lungs (Mehrad et al., 1999; Cenci et al., 2001). They participate in neutrophil recruitment into airways, and fungicidal activity of inflammatory cells during exposure to *A. fumigatus* spores (Mehrad et al., 1999; Schelenz et al., 1999; Cenci et al., 2001). A single intratracheal dose of 5×10^6 spores of *A. fumigatus* induced an inflammatory cell response in the mouse airways (Hogaboam et al., 2000), even though the response appeared more slowly than that seen for *A. versicolor*. The specific feature of the inflammation caused by *A. versicolor* was the slower onset of the TNFα response than by the other microbes, and the inflammation also lasted for a longer time than that induced by another fungus, *P. spinulosum*. This may be due to a slower rate of phagocytosis of the spores of *A. versicolor*. It has been shown that the spores of *A. fumigatus* can resist elimination by producing substances, which have an inhibitory effect on phagocytosis (Robertson et al., 1987). The results from the present study suggest that *A. versicolor* may be cytotoxic even below the level causing a detectable inflammatory response. The cytotoxicity was indicated by the mild increases in total protein and LDH in BALF. The lowest observed effect level (LOEL) was 1×10^5 spores (Table 7). The lowest biologically active dose remained unknown for *A. versicolor*. It may be considered to have a lower potential to cause inflammation than *S. californicus* or *M. terrae*, when the larger size of the spores and that the fungus did not induce expression of iNOS or cause long-lasting inflammation were taken into account.

P. spinulosum was clearly the weakest microbe to induce inflammation and toxic effects after a single spore dose (Table 7). Even though the cytokine responses were detected, the inflammatory cell response was modest and the changes disappeared rather quickly. Presumably the spores were phagocytized and processed further within a rather short time, as was the case after airway exposure to the spores of *Penicillium chrysogenum* (Cooley et al., 1999). Thus, the innate host defense was able to clear the spores without the marked support provided by the early non-adaptive immune host response. Moreover, *P. spinulosum* is not known to produce potent toxins, which may partly explain the observed low toxicity. The spores of *P. chrysogenum* have also been shown to increase the production of TNFα in the mouse lungs after a single intranasal dose (Cooley et al., 1999). Repeated dosing of the viable spores (6 weekly doses of 10^4 spores) of *P. chrysogenum* into the mouse lungs provoked Th2-cytokine-mediated inflammation, including airway easinophilia and IgE production (Cooley et al., 2000). No recruitment of eosinophils or lymphocytes into the airways was observed after a single dosage in the present study. The lowest observed effect level (LOEL) was 5×10^6 spores.

The major differences in the responses between the tested microbial species were their ability to provoke IL-6 production, induce the expression of iNOS, and cause inflammatory cell recruitment into the airways. Although all of the microbes tested provoked rapid TNFα and IL-6 responses, the IL-6 response induced by *S. californicus* was exceptionally strong. This is of interest since IL-6 has been suspected to play a critical role in the progress of lung inflammation/injury induced by exposure to environmental air pollutants (Yu et al., 2002). The spores of *S. californicus* also provoked the strongest acute inflammatory cell response. Recruitment of neutrophils, monocytes and lymphocytes may also contribute to the appearance of the adverse effects. The differences in IL-6 response and inflammatory cell recruitment were the most remarkable between *S. californicus* and *P. spinulosum*. Even at the 80-fold volumetric dose and 2,5-fold particle dose level, the *P. spinulosum* exposure provoked only a mild inflammation compared to the lower dose of *S. californicus*. The differences in the inflammatory cell response suggest that these microbes have marked differences in their ability to induce chemokine production.

Although TNFα is an important proinflammatory mediator, and it can induce production of many other mediators (Barnes et al., 1998; Luster et al., 1999), the observed TNFα responses in the airways were not necessarily associated with strong IL-6 production, inflammatory cell response, or expression of iNOS. For example, even though *P. spinulosum* provoked a relatively high peak concentration of TNFα, the IL-6 and the inflammatory cell responses were mild, and iNOS expression was not detected at all. Thus, it is obvious that in addition to TNFα, several other factors and regulators determined these responses in the mouse lungs during microbial exposure. Neither fungal species induced iNOS expression even though the acute cytokine responses were detected. Whether this is a more general difference between fungi and bacteria remains to be determined.

In the current study, LDH represented one parameter of cytotoxicity. Similarly, the presence of high total protein levels in BALF may also indicate cytotoxicity. The most of the total protein responses caused by the studied microbes consisted of albumin leakage from blood. However, also other proteins were excessively present in the airways during the airway inflammation. The origin of the other proteins remains unknown, but they may be derived from dead cells or as an exudant from blood. At least a part of the protein was derived from dead cells, as indicated by LDH. In severe inflammation, all three parameters (albumin, total protein, LDH) were increased, but the most sensitive marker was the elevated total protein level in BALF. Hence, it is not possible to separate "direct" toxicity from the inflammation-associated changes. However, the time scale of the detected biochemical and inflammatory cell responses as well as histopathological changes suggest that the major part of changes indicative of cytotoxicity were inflammation-mediated, this being especially true for *M. terrae*.

One important and intriguing question is whether the microbial airway exposure leads to systemic effects. The exposure to *M. terrae*, *S. californicus*, and *A. versicolor* provoked also systemic effects after a single dosage. *M. terrae* caused reactive changes in lymph nodes, indicating that the inflammatory response was also seen outside the lungs. *S. californicus* and *A. versicolor* induced elevated IL-6 levels in blood suggesting that they may cause also systemic effects. However, the *A. versicolor*-induced response was detected only in a very high volumetric dose (1×10^8 spores).

6.3 Responses after repeated dosing of *S. californicus* (V)

Repeated airway exposure to the spores of *S. californicus* provoked a complex immunological response. In addition to severe inflammation in the lungs, also a decrease in the amount of the splenocytes was observed. In lymph nodes, immunostimulation was detected at the lowest spore dose (2×10^3 spores). The dose response patterns in the spleen, lymph nodes and even in the lungs resembled each other indicating that the exposure-induced effects were not limited to the lungs (see V, figures 2 - 4). The severe inflammation in the lungs was observed at the dose of 2×10^7 spores, and the immunotoxic effect was seen especially in the spleen at the dose of 2×10^5 spores.

The immunostimulatory ability of the spores of *S. californicus* was already known from previous *in vivo* and also *in vitro* studies, but the immunotoxic feature has not been observed previously. The ability of streptomycetes to produce a wide variety of secondary metabolites, and special features of some of these substances may explain the observed effects. Streptomycetes produce several agents used in cancer chemotherapy. These agents include anthracyclines, such as daunorubicin and doxorubicin (Arcamone, 1998). The toxic effects of these substances on splenocytes have been demonstrated in several studies (Fornasiero *et al.*, 1992; Zaleskis *et al.*, 1995; Ferraro *et al.*, 2000). These agents have been shown to cause a rapid and massive depletion of T and B lymphocytes, especially in the spleen, moderately in the lymph nodes, and to a lesser extent in thymus, after a single low-level i.p. injection in mice (Fornasiero *et al.*, 1992; Ferraro *et al.*, 2000). Opposite immunomodulating effects have been observed also during mycotoxin exposure (Bondy and Pestka, 2000). In addition to immunostimulation, these agents may also cause immunosuppression. The immunotoxic effects provoked by some mycotoxins (e.g. gliotoxin, ochratoxin A, trichothecenes) can also affect the splenocytes.

In summary, these observations suggest that opposing effects may contribute to the responses induced by the spores of *S. californicus*. Even the lowest spore dose (2×10^3) induced a weak but detectable cellular response in the lungs and lymph nodes. The mid-dose (2×10^5) seemed to abolish the immunological stimulation presumably due to immunosuppressive or other immunotoxic effects. The highest spore dose (2×10^7) caused severe inflammation by stimulating both innate and adaptive immune responses in the lungs.

At this dose, the immunostimulation was also reflected in the reactive changes in the lymph nodes observed in histopathological analysis. The strong immunological response might partly overcome the immunotoxic effects on the spores in the spleen and lymph nodes. In addition, the repeated exposure to *S. californicus* induced an untypical lymphocyte subpopulation in the lungs. This could well be a similar population that was observed in the lungs of the same mouse strain during infection with the intracellular pathogens, *Chlamydia pneumoniae*, or influenza A virus (Penttilä *et al.*, 1998).

In this mouse model, the overall no observed effect level (NOEL) after repeated exposure was below 2×10^3 spores/week, and with respect to the immunotoxic effect in the spleen, the dose level was below 2×10^5 spores/week. A sufficient dose to cause strong airway inflammation in mice was between 2×10^5 and 2×10^7 spores/week.

Repeated dosing of *S. californicus* (2×10^7 spores) provoked a markedly stronger inflammatory cell response in the airways than that observed in a single dose study at any tested dose (2×10^7, 1×10^8 or 3×10^8 spores) (I). The cell profiles were rather similar in both studies: Neutrophils were the most prominent cells in BALF but also macrophages and lymphocytes were recruited into the airways. Moreover, the total protein, albumin and LDH responses were stronger after repeated dosing indicating stronger inflammatory and toxic responses. The albumin and LDH responses induced by 2×10^7 spores in the repeated dose study were even stronger than the responses provoked by 1×10^8 spores in the single dose study (I).

However, not all of the responses were stronger after repeated dosing of the spores. IL-6 response seemed to be weaker or more transient after repeated dosing than after single dosing of *S. californicus*. In addition, iNOS expression was not detectable after repeated dosing. These differences may be due to a change from an acute inflammation to a more chronic type.

The effects after repeated dosing of the spores of *S. californicus* occurred at the same level of exposure (based on spore numbers) as seen with toxic spores of *Stachybotrys chartarum* in the mouse lungs (Nikulin *et al.,* 1997). *Stachybotrys chartarum* is one of the most widely studied toxigenic fungus of the moisture-indicator microbes. Repeated intranasal dosing of

10^5 spores (6 doses over 3 weeks) caused histopathologically detectable inflammation in mouse lungs (Nikulin et al., 1997). The reaction was more severe with the spores of the fungal strain capable of producing satratoxins and higher amounts of other mycotoxins. Repeated dosing of 1×10^3 such spores induced inflammation in contrast to the spores of a less toxic strain. In addition, it has been shown that spores of *Stachybotrys chartarum* extracted with methanol to remove active secondary metabolites are markedly less potent at provoking acute inflammation, cytotoxicity and haemorrhage in the rat lungs (Rao et al., 2000a; 2000b). These observations indicate that toxic secondary metabolites are important in causing inflammatory responses at least during *Stachybotrys chartarum* exposure.

6.4 Comparison of *in vivo* and *in vitro* responses (I - IV)

The present *in vivo* results are well in concordance with the data obtained in cell cultures *in vitro* by the same microbial species. *S. californicus* and *M. terrae* (BA26) have been shown to cause intense production of inflammatory mediators in mouse macrophages (Hirvonen et al., 1997a, 1997b, 1997c; Huttunen et al., 2000). *S. californicus* induced significant TNFα and IL-6 responses in these cells between 4 and 8 hours exposure (Hirvonen 1997a). Moreover, iNOS protein was detectable from 8 hours to the end of the experiment (24 hours), and a marked increase in nitrite concentration, an indicator of increased NO production, was observed from 16 hours to the end of the experiment (at and above 1×10^5 spores). Severe cytotoxicity was not observed *in vitro* during the exposure at the tested doses. The spores of *S. californicus* induced the production of reactive oxygen species (ROS) in both human polymorphonuclear leucocytes and mouse macrophages *in vitro* (Ruotsalainen et al., 1995; Hirvonen et al., 1997c). This may partly explain the observed cytotoxicity.

M. terrae provoked immunological responses in a slower kinetics compared to *S. californicus* in mouse macrophages *in vitro* (Huttunen et al., 2000). *M. terrae* induced cytokine responses after 4 hours exposure (5×10^6 bacterial cells). Inducible NOS was detectable from 16 hours up to 48 hours, and a marked increase in nitrite concentration, as an indicator of increased NO production, was observed already from 8 hours to the end of the experiment. Marked cytotoxicity was observed *in vitro* only after exposure to the highest tested dose of *M. terrae* (5×10^7 cells/ml). These observations indicate that the same mediators are induced *in vivo*, even though the time frame is somewhat different. Cytokine responses were observed slightly

earlier *in vivo*, but the iNOS expression was detectable at later time points. *M. terrae* was not highly cytotoxic *in vitro*, an observation which was corroborated *in vivo*. These results suggest that alveolar macrophages are important cells in the manifestation of the *in vivo* responses provoked by *S. californicus* and *M. terrae*, but are likely not the only responding cells.

In line with the *in vivo* observations, *S. californicus* induced stronger TNFα, IL-6 and NO responses in mouse macrophages *in vitro* than the fungal species *A. versicolor* and *P. spinulosum* at the doses with equal particulate numbers (1×10^5, 1×10^6, and 1×10^7 spores) (Huttunen et al., in press). Fungi-induced IL-6 and NO production were hardly detectable, in contrast to the very strong responses produced by the *S. californicus*. *A. versicolor* provoked a stronger TNFα response than *P. spinulosum* at the doses below 1×10^7 spores *in vitro*. The highest dose of *P. spinulosum*, instead, provoked a much stronger TNFα response compared to a similar dose of *A. versicolor in vitro*. A similar pattern was evident also *in vivo*. The *in vitro* observation gives further support to the concept of overloading, i.e. it is possible that the spores of *P. spinulosum*, since they are larger particles, can cause strong production of TNFα due to a nonspecific response via overloading of the phagocytes. *P. spinulosum* has been shown to be a poor inducer of the production of inflammatory cytokines in lavaged rat alveolar macrophages *in vitro* (Shahan et al., 1998).

S. californicus also caused cytotoxicity in mouse macrophages *in vitro* at the lower dose level than the fungal species (Huttunen et al., in press), which is corroborated by the present *in vivo* findings. A marked increase in the cytotoxicity provoked by the fungal species *in vitro* was detected at the highest dose (10^7 spores) (Huttunen et al., in press), which may partly be due to the overloading phenomenon in those conditions. Since no IL-6 response was detected after exposure to *A. versicolor* or *P. spinulosum* in mouse macrophages *in vitro* (Huttunen et al., in press), this suggests that cells other than macrophages contribute to the response in the mouse or perhaps the regulation of IL-6 production is more complex *in vivo*.

These data together indicate that mouse macrophages *in vitro* predict well the acute cytokine and cytotoxic responses of these microbes *in vivo*. This suggests that mouse macrophages are suitable in screening for the inflammatory and cytotoxic potential of moldy house microbes.

6.5 Clinical implications

The observations that the indicated microbes cause inflammation *in vivo* suggest that they can contribute to inflammation associated adverse health effects experienced by the occupants of moisture-damaged buildings. An immunotoxic effect, as observed in mice after repeated dosing of the spores of *S. californicus*, may interfere with the normal functions of the host immune system and increase the risk for infections caused by other microorganisms. However, it is not possible to make any direct quantitative extrapolations from the present findings to exposure situations in humans in moisture-damaged buildings. It is not known which level of exposure is sufficient to cause symptoms in humans. On the other hand, the present studies did not reveal the lowest harmful exposure levels. Further studies are needed to answer these questions. The species differences in sensitivity have also to be considered. In addition, many important factors influencing the harmfulness of a specific microbe still have to be determined. For example, the growth conditions of a microbe may affect its potency to be harmful for the host (Nielsen et al., 1998; Nunez et al., 2000; Murtoniemi et al., 2001), as well as the presence of other microbes growing in the same conditions (Marin et al., 1998; Picco et al., 1999). Moreover, the occupants of moisture-damaged buildings are exposed to several agents and microbes simultaneously, and there may be marked interactions between different toxins or other substances. It has been shown that cell wall components, bacterial LPS and fungal β(1,3)-D-glucan have synergistic effects to cause pulmonary inflammation (Fogelmark et al., 1994). Mycotoxins as well as MVOC's may also have interactions, which potentiate their toxicity (Dowd et al., 1989; Korpi et al., 1999; Carlson et al., 2001). Pollutants in the ambient air may also have synergistic effects with moisture-damage derived hazardous substances. However, currently very little is known about these interactions. In addition, individual differences have a marked influence on the risk of adverse health effects caused by exposure to microbes or their products in moisture-damaged buildings. These aspects should also be taken into account when any risk assessment concerning the moldy building phenomenon is being undertaken.

7 CONCLUSIONS

The following conclusions can be drawn from the present study.

1. The studied microbes had a different potential to cause inflammatory and toxic responses in the lungs after single dosing into the airways. Major differences were observed in their ability to recruit inflammatory cells into the airways, and to induce IL-6 and iNOS production. The bacterial species *S. californicus* and *M. terrae* had approximately equal potency to evoke these responses. *S. californicus* provoked strong acute inflammation, whereas *M. terrae* caused sustained inflammation. *A. versicolor* was more potent at inducing inflammation than the other fungus, *P. spinulosum*. When compared based on comparable volumetric doses, the bacterial species *S. californicus* and *M. terrae* were more potent at inducing adverse effects than the fungal species *A. versicolor* and *P. spinulosum*. Based on these data, *P. spinulosum* was the weakest inducer of inflammation and toxicity.

2. Airway exposure to these microbes can provoke also systemic effects. Both *M. terrae* and *S. californicus* provoked reactive changes in the lymph nodes, and *S. californicus* caused immunotoxicity especially in the spleen. Moreover, exposure to the spores of *S. californicus* increased the TNFα and IL-6 levels in blood.

3. Repeated dosing of *S. californicus* potentiated but also sometimes repressed immune responses compared to the single dosing. Inflammatory cell recruitment into the lungs was markedly enhanced whereas IL-6 and iNOS responses were depressed with repeated dosing. After repeated dosing, signs of the inflammatory response in lungs were detectable even at a very low exposure level.

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I

Jussila, J., Komulainen, H., Huttunen, K., Roponen, M., Hälinen, A., Hyvärinen, A., Kosma, V.-M., Pelkonen, J., and Hirvonen, M.-R. Inflammatory responses in mice after intratracheal instillation of spores of *Streptomyces californicus* isolated from indoor air of a moldy building. Toxicol. Appl. Pharmacol. 2001;171:61-69.

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V

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