INFLAMMATORY AND CYTOTOXIC POTENTIAL OF SELECTED MOLDY HOUSE MICROBES IN VITRO

Kati Huttunen

National Public Health Institute
Department of Environmental Health
P.O. Box 95, FIN-70701 Kuopio, Finland

and

University of Kuopio
Department of Environmental Sciences
P.O. Box 1627, FIN-70211 Kuopio, Finland

ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Natural and Environmental Sciences of the University of Kuopio for public examination in Auditorium L1, Canthia building,
University of Kuopio, on Friday 6th of June 2003, at 12 o’clock noon.
Publisher: National Public Health Institute
Mannerheimintie 166
FIN-00300 Helsinki, Finland
Phone +358 9 47441
Telefax +358 9 47448408

Author's address: National Public Health Institute
Department of Environmental Health
P.O. Box 95, FIN-70701 Kuopio, Finland
Phone +358 17 201320
Telefax +358 17 201265
E-mail Kati.Huttunen@ktl.fi

Supervisors: Docent Maija-Riitta Hirvonen, Ph. D.
National Public Health Institute
Kuopio, Finland
Docent Aino Nevalainen, Ph. D.
National Public Health Institute
Kuopio, Finland
Marja-Leena Katila, M.D.
Kuopio University Hospital
Kuopio, Finland

Reviewers: Docent Harri Alenius, Ph. D
Finnish Institute of Occupational Health
Helsinki, Finland

Docent Kaija Linnainmaa, Ph. D
Finnish Institute of Occupational Health
Helsinki, Finland

Opponent: Professor Pentti Huovinen
National Public Health Institute
Turku, Finland

ISSN 0359-3584
ISBN (pdf-version) 951-740-365-8
ISSN (pdf-version) 1458-6290

Kuopio University Printing Office, Kuopio, Finland, 2003

ABSTRACT

Both dampness and subsequent mold growth have been associated with adverse health effects in the occupants of the damaged buildings, but the etiological factors and mechanisms behind the multitude of symptoms experienced by the occupants are still to be clarified. In addition, interactions between the different exposures are possible, though this has not been extensively studied in the moisture damaged environment.

The inflammatory and cytotoxic potencies of selected indoor air microbes were assessed by measuring the production of inflammatory mediators such as interleukin (IL)-1β, IL-6, IL-10 tumor necrosis factor (TNF)-α, nitric oxide and reactive oxygen species, as well as cytotoxicity in human and mouse cell lines after exposure to graded doses of the bacteria Mycobacterium avium, Mycobacterium scrofulaceum, Mycobacterium terrae, Streptomyces californicus, Bacillus cereus, Pseudomonas fluorescens and fungi Stachybotrys chartarum, Aspergillus versicolor and Penicillium spinulosum. In addition, the different cell lines (human 28SC and mouse RAW264.7 macrophages, human A549 lung epithelial cells) were compared to evaluate the consistency of the findings in different in vitro models.

In these studies, the bacteria were found to be more potent than the fungi at inducing inflammatory responses, and importantly, the so-called indicator microbes of moisture damage, P. fluorescens and Str. californicus proved to be the most toxic bacteria, whereas S. chartarum was the most toxic fungi. In comparison of the different cell lines, the human cell lines were less sensitive to the toxic effects and they produced significantly lower levels of inflammatory mediators compared to the mouse macrophages. However, all cell lines responded essentially to the same microbial exposures; P. fluorescens and Str. californicus. With respect to the studied inflammatory markers, the pro-inflammatory cytokine IL-6 was the most consistent in all cell lines. The association between the produced metabolite profile and the toxicology of the fungal strains was studied by analyzing the metabolites produced by 20 Stachybotrys strains, whose inflammatory and toxic effects had been studied earlier. The isolated metabolites were further tested for their inflammatory and cytotoxic abilities, revealing that the toxicity of a particular strain was associated with the produced metabolites, although the inflammatory potency of non-toxic strains could not be clarified. Finally, the interactions between different microbes in co-exposure were studied by exposing mouse macrophages to Mycobacterium terrae, Bacillus cereus, Pseudomonas fluorescens, Aspergillus versicolor, Stachybotrys chartarum and Penicillium spinulosum alone and together with a low dose of Streptomyces californicus. Out of the studied microbes, S. chartarum caused a synergistic increase in the production of IL-6. The interaction between these microbes was studied further by simultaneous exposure with Str. californicus combined with the common mycotoxins produced by Stachybotrys spp. There was a synergistic increase in TNF-α and IL-6 production, and cytotoxicity after co-exposure to Str. californicus and trichodermia. Exposure to Str. californicus increased the nuclear localization of the transcription factor NF-κB, but the synergistic increase in IL-6 production could not be explained by the amount of NF-κB in the nucleus.

In summary, these data indicate that the information obtained from in vitro experiments can be utilized both in screening studies for further research and as a basis for detailed mechanistic studies. However, the findings of in vitro studies have to be confirmed both in vivo and with epidemiological data in order to conduct an appropriate risk assessment.
To my angel,
Kari
ACKNOWLEDGEMENTS

The present study was carried out in the Department of Environmental Health, National Public Health Institute, Kuopio during the years 1998-2003. I would like to thank the Director of the Department, Professor Jouko Tuomisto and the Head of the Laboratory of Toxicology, Docent Hannu Komulainen for providing the facilities for this study.

My heartfelt gratitude belongs to my principal supervisor Docent Maija-Riitta Hirvonen for her firm belief in me and this project. Her enthusiasm and expertise have been an invaluable help whether the problem relates to gardening, shoes or science. I am also grateful to my other supervisors Docent Aino Nevalainen and Marja-Leena Katila, M.D. for their insightful discussions, positive attitude and support during this work.

My sincere thanks are due to the official referees of my thesis, Docent Harri Alenius and Docent Kaija Linnavuori for their well-thought-out comments and constructive criticisms. I also thank Ewen MacDonald, Ph. D., for revising the language of the original articles and of this thesis, including a total of 107 missing or misplaced definite articles.

My dear colleagues Juha Jussila, Ph. D and Marjut Roponen, Ph. D both induce a statistically significant increase in gratefulness, thus my warm thanks for the friendship, moral support and fun years together! I want to thank also Timo Murtoniemi, M. Sc for the team spirit despite the long distance between our office rooms.

I am grateful to Ms. Virpi Koponen, the late Ms. Tuula Wallenius, Ms. Leena Heikkinen and Ms. Heli Martikainen for teaching me the essentials of laboratory work and for helping me with the analysis of numerous samples, and Ms. Mirja Ojainvääli, Ms. Reetta Tiihonen and Ms. Arja Rönkkö for their excellent technical assistance. Our group has been a very pleasant place to work, thanks to the numerous coffeebreaks with delicacies...

The contribution of my co-authors Marjo Ruotsalainen, Eila Torvinen, Pirjo Torkko, Anne Hyvärinen, Kristian Fogg Nielsen, Birgitte Andersen and Bruce Jarvis has been essential for this work. In particular, I appreciate the fruitful collaboration with the Laboratory of Microbiology, and the mold group in Denmark. My thanks are sent also to Mikko Vahteristo, M. Sc and Pekka Tiittanen, M. Sc. for their help and advice regarding the difficult discipline of statistics.
The entire personnel of the Department of Environmental Health and the library of the Neulanen Research Center have been the heart of a friendly working environment. Thanks to the unofficial KTL Sähly team, I have been able to participate in some form of team exercise.

Whenever I have been in danger of taking myself too seriously, my beloved friends have been there for me, reminding me of the things that are really important. In addition, my personal trainers Tiltu and Tipsi have taken excellent care of my physical and mental health during this work.

I wish to thank my parents Maija and Simppa for their love and support throughout my life. I could not think a better way to grow up than with my funny family. My precious little sister Sanna is the light of my life and my absolute VIP. I also thank my grandmother Hilja for naming me "Kesänkeiju". Finally, I owe a lot to missa chickabee Antti; you complete me.

This study was financially supported by The Academy of Finland, Finnish Research Programme on Environmental Health, Graduate School in Environmental Health and the Juho Vainio Foundation.

Kuopio, May 2003

Kati Huttunen
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>28SC</td>
<td>human macrophage cell line</td>
</tr>
<tr>
<td>A549</td>
<td>human lung epithelial cell line</td>
</tr>
<tr>
<td>AFB</td>
<td>aflatoxin B1</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>CAM</td>
<td>cell adhesion molecule</td>
</tr>
<tr>
<td>cfu</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>DAS</td>
<td>diacetoxycirpenol</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DON</td>
<td>deoxynivalenol</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FB</td>
<td>fumonisin B</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GC-MS/MS</td>
<td>gas-chromatography-mass detector</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HIV-1</td>
<td>human immunodeficiency virus -1</td>
</tr>
<tr>
<td>I-κB</td>
<td>inhibitory-κB</td>
</tr>
<tr>
<td>IFNγ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible NO synthase</td>
</tr>
<tr>
<td>LAM</td>
<td>lipoarabinomannan</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>LTA</td>
<td>lipoteichoic acid</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-tetrazolium bromide</td>
</tr>
<tr>
<td>MVOC</td>
<td>microbial volatile organic compound</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor -κB</td>
</tr>
<tr>
<td>NIV</td>
<td>nivalenol</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen associated molecule pattern</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognizing receptor</td>
</tr>
<tr>
<td>RAW264.7</td>
<td>mouse macrophage cell line</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid (mRNA = messenger RNA)</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>T-2</td>
<td>T-2 toxin</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>ZEA</td>
<td>zearalenone</td>
</tr>
</tbody>
</table>
LIST OF ORIGINAL PUBLICATIONS

This thesis contains material from the following original publications, referred to in the text by the Roman numerals (I-V).


CONTENTS

1. INTRODUCTION........................................................................................................... 15
2. REVIEW OF THE LITERATURE.................................................................................... 16
   2.1 Adverse health effects associated with moisture damaged buildings.............. 16
   2.2 Exposure agents in moisture damaged buildings............................................ 17
      2.2.1 Microbes ........................................................................................................ 17
      2.2.2 Microbial components ................................................................................. 19
      2.2.3 Microbial metabolites .................................................................................. 20
      2.2.4 Allergens ...................................................................................................... 21
   2.3 Interaction between different exposures......................................................... 21
      2.3.1 In vitro studies on interactions of mycotoxins ........................................... 22
      2.3.2 In vivo studies on interactions of mycotoxins ............................................ 24
      2.3.3 Effect of mycotoxins on immunoresistance .............................................. 24
   2.4 Inflammation ..................................................................................................... 27
      2.4.1 Cells involved in inflammation .................................................................. 27
      2.4.2 Mediators of inflammation ........................................................................ 29
      2.4.3 Receptors associated with microbe recognition ......................................... 32
      2.4.4 Signal transduction ...................................................................................... 33
3. THE AIMS OF THE STUDY......................................................................................... 36
4. MATERIALS AND METHODS..................................................................................... 37
   4.1 Exposure agents .................................................................................................. 37
      4.1.1 Microbes ...................................................................................................... 37
      4.1.2 Fungal metabolites ...................................................................................... 37
   4.2 Cell cultures ........................................................................................................ 40
      4.2.1 RAW264.7 Mouse macrophages ................................................................... 40
      4.2.2 A549 Human lung epithelial cells ................................................................ 40
      4.2.3 28SC Human macrophages ......................................................................... 40
   4.3 Study design ......................................................................................................... 41
      4.3.1 Exposure ...................................................................................................... 41
      4.3.2 Dose-response and time course studies ..................................................... 41
   4.4 Biochemical analyses .......................................................................................... 44
   4.5 Statistical analyses .............................................................................................. 44
5. RESULTS........................................................................................................................................45
   5.1 Differences between cell lines..................................................................................................45
      5.1.1 Production of inflammatory mediators 45
      5.1.2 Cytotoxicity 45
   5.2 Differences between microbes..............................................................................................47
      5.2.1 Production of inflammatory mediators 47
      5.2.2 Cytotoxicity 47
   5.3 Differences between fungal metabolites..................................................................................49
      5.3.1 Production of inflammatory mediators 49
      5.3.2 Cytotoxicity 49
   5.4 Synergistic interaction..............................................................................................................50
      5.4.1 Production of inflammatory mediators 50
      5.4.2 Cytotoxicity 50
      5.4.3 Nuclear binding activity of NF-κB 50

6. DISCUSSION...................................................................................................................................54
   6.1 Characteristics of the studied cell lines...................................................................................54
   6.2 Validity of the selected doses..................................................................................................56
   6.3 Differences in the inflammatory and cytotoxic potency of microbial exposures 57
   6.4 Microbial interactions..............................................................................................................59
   6.5 The value of in vitro-studies in environmental toxicology......................................................60

7. CONCLUSIONS...........................................................................................................................61

8. REFERENCES...............................................................................................................................62

ORIGINAL PUBLICATIONS
1. INTRODUCTION

In today’s environment many microbes have adapted to the living conditions provided by the construction materials in the buildings in which we live and work. The co-existence of environmental microbes and man is not always harmonic, sometimes it can lead to a conflict of interest, even a full scale war, complete with chemical agents and offensive strategies.

One of the key elements necessary for microbial growth in buildings is a sufficient supply of water, either due to condensation, capillary rise or accidental leaks from plumbing systems or roofs (Nevalainen et al. 1998). Both dampness and subsequent mold growth have been associated with adverse health effects in the occupants of the moisture damaged buildings, but the etiological factors and mechanisms behind the multitude of symptoms are still unclear (Bornehag et al. 2001).

Many of the symptoms and diseases associated with exposure to a moisture damaged environment are of inflammatory origin, or related to impairment of immune defense (Dales et al. 1991). Thus, the inflammatory potency and toxicity of the indoor air microbes is a relevant basis for comparison between different exposures. There is already evidence for differences in the immunogenic potential of various components of microbes (Jagielo et al. 1996, Fogelmark et al. 2001), as well as the toxicity of the microbial metabolites (Hussein and Brasel 2001). In a moldy environment, exposure is multifactorial, consisting of several groups of microbes and their metabolites, and/or potentially toxic compounds released into the air as a consequence of microbial destruction of building materials. It is possible that there could be interactions between different exposure agents, though this has not been extensively studied in the moisture damage situation. Altogether, there is a paucity of comparative toxicological data about moldy house microbes and their combined effects.

Exposure to foreign agents induces cellular responses which are the initial event leading eventually to the reported symptoms. In vitro studies may represent an over-simplified model of the whole organism, but they do offer a reproducible and cost-effective way to study the potential risk factors among the exposure agents. The information obtained from cell lines can be utilized both in screening studies for further research and a basis for detailed mechanistic studies. In addition, the in vitro studies are an important link between responses seen in animal models and human clinical studies, which are all needed for proper risk assessment.
2. REVIEW OF THE LITERATURE

2.1 Adverse health effects associated with moisture damaged buildings

Dampness and moisture in buildings and the associated adverse health effects have been increasingly studied during the last two decades. Epidemiological evidence indicates that living or working in damp buildings increases the risks for adverse health effects in the respiratory tract. In addition to the airway symptoms such as cough, wheeze and asthma, also nonspecific symptoms including tiredness and headache, and frequent respiratory infections have been linked with living in moisture damaged indoor environments (Peat et al. 1998, Bornehag et al. 2001). Similarly the exposure defined as microbial growth indoors has been associated with both reported and measured clinical symptoms, although this evidence is less conclusive due to the inconsistent exposure assessment (Verhoeff and Burge 1997, Burr 2001).

One important technique of determining a causal relationship between exposure to molds and adverse health effects is the intervention study. Such studies generally indicate that the symptoms decrease or disappear when the moisture damage is repaired or the occupants are relocated from the water-damaged environment (Sudakin 1998, Ahman et al. 2000, Meklin 2002). In line with the intervention studies, a decrease in symptoms has been observed during absence from the moisture damaged working environment and correspondingly the symptoms reappear when the employees return to work in the moldy environment (Hirvonen et al. 1999).

The most common adverse health effects associated with moisture damaged buildings can be grouped into irritative and nonspecific symptoms, respiratory infections, allergic diseases and chronic pulmonary diseases (Husman 1996). Irritative symptoms, typically located in the upper respiratory tract include cough, wheeze, sore throat, and nasal congestion and discharge (Platt et al. 1989, Brunekreef et al. 1992). Nonspecific symptoms include fatigue, nausea, headache and difficulties in concentration (Peat et al. 1998, Koskinen et al. 1999). The increase in respiratory infections has been reported both in children and adults, with manifestations of the infection ranging from common cold to recurrent sinusitis, rhinitis and acute bronchitis (Brunekreef 1992, Koskinen et al. 1995, Pirhonen et al. 1996). Allergy-like symptoms have been associated with moisture damaged environments, although the
prevalence of IgE mediated mold allergies was found to be rare (5-10 %) in a recent study (Taskinen et al. 2001). The prevalence did not differ from the estimated prevalence in the general population (6 %) (Kurup et al. 2000). Consequently, IgE mediated allergic reactions are considered as less important than nonspecific inflammation induced by microbial exposure. Nevertheless, the adverse health effects of exposure to moisture damaged environment may be more prominent and occur more frequently in atopic and asthmatic individuals (Carrer et al. 2001). Chronic pulmonary diseases such as chronic allergic alveolitis, organic dust toxic syndrome (ODTS) and chronic bronchitis are often attributed to extensive exposure to microbes in occupational environments (Patel et al. 2001). Some reports indicate that similar syndromes may be associated with moisture damaged buildings (Seuri et al. 2000). Recent findings also suggest that exposure to moisture damaged environment may lead to autoimmune diseases, as a cluster of rheumatic diseases has been found in employees in a moisture damaged workplace (Myllykangas-Luosujärvi 2002, Roponen et al. 2001).

2.2 Exposure agents in moisture damaged buildings

Excess moisture, resulting from leaking roofs or plumbing, poor ventilation or flaws in construction of the building, provides a habitat for microbial growth indoors. As a consequence, such an environment contains an excessive amount of microbes and their byproducts. In addition to the microbial cells, individuals living in a moldy house are exposed to a variety of biological substances including spores, fragments of the mycelium, structural components, volatile organic compounds, and microbial metabolites (Hyvärinen 2002).

2.2.1 Microbes

The generally used term “mold” refers to visible fungal growth. In addition to mycelia of filamentous fungi, the mold may contain also yeasts and bacteria such as actinomycetes. Microbial spores are present practically everywhere, originating from outdoor air and indoor sources like foodstuffs or plants. Consequently, almost any material may act as a substrate for microbial growth if the moisture content is appropriate (Gravesen et al. 1994). The concentrations of viable airborne fungi in residences vary between $10^3$ - $10^5$ cfu/m$^3$, whereas the concentrations of viable bacteria range between $10^1$ - $10^4$ cfu/m$^3$. There are considerable
spatial and temporal variations in the indoor air concentrations of microbes, depending on the activity of the occupants and characteristics of the household (Hyvärinen 2002). Moreover, the culturable portion of microbes may be as little as 1% of the total number actually present (Gravesen et al. 1994). Some microbes are considered as being indicative of moisture damage due to their occurrence in problem buildings; these include yeasts, fungi like *Aspergillus fumigatus, Aspergillus versicolor, Exophiala, Fusarium, Phialophora, Stachybotrys, Trichoderma, Ulocladium*, and bacterial species like actinobacteria and non-enteric gram negative bacteria. These have been shown to often grow in mold-infested materials, but they do not belong to the normal flora of the indoor environment (Samson et al. 1994).

Among the indoor microbial species selected to be studied in this thesis, four belong to the indicator species, two were selected to represent normal microbial flora and one because of the spectrum of interesting characteristics which it possesses. The fungus *Aspergillus versicolor* is both an indicator fungus of mold problem in a building, and a common toxin producer. It is also an opportunistic pathogen, capable of infecting the lungs if the host defense of the exposed individual is suppressed (Pitt 1994, Samson et al. 1994). *Stachybotrys chartarum* is another indicator microbe, and notorious for its ability to produce potent toxins (Gravesen et al. 1994). The growth of this fungus in water-damaged buildings has been associated with adverse health effects such as hematological, respiratory and other symptoms although no exact causal link has been established (Johanning et al. 1996, Gordon et al. 1999, Johanning et al. 1999). The third studied fungus *Penicillium* spp. has been detected in moisture damaged buildings, but they also belong to the common airborne fungal flora in indoor environments (Hyvärinen et al. 1993, Flannigan and Morey 1996). Thus the presence of *Penicillium* species is not considered as an indication of mold damage unless their numbers are abnormally high, which indicates that there is an amplification site of *Penicillium* in the indoor environment.

The bacterial species generally associated with moisture damage are actinomycetes (*Streptomyces* spp.) and non-enteric gram negative bacteria (e.g. *Pseudomonas* spp.). *Streptomyces californicus* is a gram positive, sporulating bacterium which has been a focus of interest for moldy house research both for its association with mold problem buildings (Nevalainen 1991) and its inflammatory potency (Hirvonen et al. 1997, Jussila et al. 1999). *Pseudomonas fluorescens* is a gram negative bacterium with lipopolysaccharide (LPS) as a cell wall component. It is a species that is common in the outdoor air but is found also in
moisture damaged buildings and poorly maintained humidifier systems (Rylander and Haglind 1984, Nevalainen 1989). The gram positive bacteria *Bacillus* spp. are not considered as indicator microbes, instead they are referred to as common environmental bacteria also present in the indoor environment. In addition, there are toxin-producing strains in this bacterial genus, and some are found in damaged building materials (Andersson et al. 1999).

Finally, there are the environmental mycobacteria, the group of microbes whose immunogenic abilities make them interesting from a toxicological point of view. They are slowly growing bacteria, most of which are nonpathogenic to humans, apart from their potential pathogenicity in immunocompromised patients. Mycobacteria have a lipid-rich cell wall, which is resistant to acidic conditions and treatments with many chemical agents. This enables them to survive in changing environmental condition, even inside of the infected host cell (Falkinham 1996). Environmental mycobacteria have been isolated from the indoor air of a moisture damaged building, but their prevalence in such environments is so far poorly characterized due to their difficult and time-consuming culturing conditions (Rautiala et al. in press).

### 2.2.2 Microbial components

In addition to microbial cells or spores, physiologically reactive agents originating from microbial growth may also be components of cell wall or other structures. These include peptidoglycans, endotoxins, lipoarabinomannans and (1→3)-β-D-glucans. Peptidoglycan is the cell wall component of both gram positive and gram negative bacteria. It has been shown that fragments of polymeric peptidoglycan may have both immunostimulating and immunosuppressing effects (Schleifer 1985). Endotoxin, also known as lipopolysaccharide (LPS) is a cell wall component of gram negative bacteria. Exposure to endotoxin is well known to induce many physiological reactions, e.g. fever, leukopenia, activation of macrophages and complement, release of prostaglandins, cytokines and nitric oxide (Rylander 1994, Jagiello et al. 1996) Lipoarabinomannan (LAM) is a typical component of the cell walls of mycobacteria and related species, but it is also found in actinobacteria (Sutcliffe 2000). This glycolipid induces immune reactions in macrophages, modulating the immunity and pathogenicity of mycobacterial infection (Venisse et al. 1995). (1→3)-β-D-glucans are immunogenic cell wall components of plants, fungi and certain bacteria. Both humoral and
cell-mediated immunity are stimulated by exposure to (1→3)-β-D-glucans, but their immunostimulating activity is weaker (Fogelmark et al. 1994, Rylander and Lin 2000).

### 2.2.3 Microbial metabolites

Microbial volatiles (MVOC) are volatile organic compounds responsible for the characteristic moldy, musty or earthy odors of microbial growth. The production of MVOCs varies according to the physiological state of the microbe, depending on the growth conditions such as availability of water, nutrients or presence of competing organisms (Gravesen et al. 1994, Sunesson 1996). Measuring the volatile products of fungi has been proposed to be useful in characterizing the moisture damaged buildings, although the production of MVOC indicates merely that there is active microbial growth in the building. Moist building materials may themselves also act as a source of VOCs, thus the presence of volatile compounds is not specific for microbial metabolism (Korpi 2001).

Mycotoxins are secondary metabolites of fungi that have toxic effects on animals and humans. They are relatively low molecular mass organic compounds, the majority of them being nonvolatile. Toxins can be detected from substrate, mycelium, spores and fragments of the hyphae (Samson 1992). Mycotoxins are capable of disrupting various cellular structures such as membranes, and interfering with vital cellular processes such as protein, RNA and DNA synthesis. The most common mycotoxins associated with human toxicoses are aflatoxins, which cause immunosuppression, and induce hepatotoxic, mutagenic, teratogenic and carcinogenic effects; ochratoxin, which is nephrotoxic, immunosuppressive, carcinogenic and teratogenic; trichothecenes, which are associated especially with immunosuppressive and hemorrhagic effects and alimentary toxic aleukia (ATA); zearalenone (ZEA) which has estrogenic effects; and fumonisins which are possibly carcinogenic. Some of the notorious mycotoxins are no longer common as a cause of human toxicoses today because of improved hygiene and food processing, e.g. ergot alkaloids, the etiologic factor causing ergotism, a disease characterized by gastrointestinal symptoms and neurotoxic effects (Peraica et al. 1999, Etzel 2002) (Table 1).
Table 1. Toxic effects of some common mycotoxins and examples of the producing fungal genus (modified from Hussein and Brasel 2001)

<table>
<thead>
<tr>
<th>Toxic effect</th>
<th>Associated toxins</th>
<th>Produced by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immuno-suppressive</td>
<td>aflatoxins, gliotoxin, ochratoxins, trichothecenes</td>
<td><em>Alternaria</em>, <em>Aspergillus</em>, <em>Fusarium</em>, <em>Myrothecium</em>, <em>Penicillium</em>, <em>Stachybotrys</em>, <em>Tricothecium</em></td>
</tr>
<tr>
<td>Hepatotoxic</td>
<td>aflatoxins, ochratoxins, sterigmatocystin</td>
<td><em>Aspergillus</em>, <em>Penicillium</em></td>
</tr>
<tr>
<td>Nephrotoxic</td>
<td>citrinin, ochratoxins, patulin, sterigmatocystin</td>
<td><em>Aspergillus</em>, <em>Fusarium</em>, <em>Penicillium</em></td>
</tr>
<tr>
<td>Neurotoxic</td>
<td>ergot alkaloids, fumonisins, gliotoxin, tremorgens, trichothecenes</td>
<td><em>Acremonium</em>, <em>Alternaria</em>, <em>Aspergillus</em>, <em>Fusarium</em>, <em>Myrothecium</em>, <em>Penicillium</em>, <em>Stachybotrys</em>, <em>Tricothecium</em></td>
</tr>
<tr>
<td>Teratogenic</td>
<td>aflatoxins, zearalenone</td>
<td><em>Aspergillus</em>, <em>Fusarium</em></td>
</tr>
<tr>
<td>Carcinogenic</td>
<td>aflatoxins, fumonisins, ochratoxins, sterigmatocystin</td>
<td><em>Aspergillus</em>, <em>Fusarium</em>, <em>Penicillium</em></td>
</tr>
<tr>
<td>Hemorrhagic</td>
<td>aflatoxins, patulin, trichothecenes</td>
<td><em>Aspergillus</em>, <em>Fusarium</em>, <em>Myrothecium</em>, <em>Penicillium</em>, <em>Stachybotrys</em>, <em>Tricothecium</em></td>
</tr>
</tbody>
</table>

2.2.4 Allergens

The indoor environment contains many allergens derived from both organic and inorganic sources. The major indoor allergens include the antigens of house dust mites, cat, dog, mouse, cockroach and some fungi. The concentrations of both house dust mite and fungal allergens are dependent on the dampness conditions; thus an increase in the level of these allergens may be related with moisture damage.

The most prevalent allergenic indoor air fungi are *Aspergillus*, *Cladosporium* and *Penicillium*. In addition to spores, they may contain allergens in their structural components like mycelia and also in their excreted materials (Ledford 1994). House dust mites are prevalent in humid climates (Iversen and Dahl 1995), whereas in a Finnish study, mites were detected only in 20% of water-damaged buildings and equally often in reference buildings (Hyvärinen et al. 1993). Mite allergen levels correlate poorly with the numbers of mites in house dust, because the main allergens are found also from degraded mites and their feces. The highest mite allergen levels are usually found in mattresses and carpets (Carrer et al. 2001).

2.3 Interaction between different exposures

In a moisture damaged building, the exposure may consist of several components enhancing or counteracting each others’ effects. In the case of a synergistic effect, the interaction leads to a total effect greater than the sum of the two effects taken independently. On the other hand,
an interaction may be antagonistic, meaning that two or more substances interact in such a way that their joint effect on living cells or tissues is decreased. Interactions between different mycotoxins have been of interest especially in agriculture, since the contamination of raw materials, products and animal feeds with toxin-producing fungi has a major impact on the economy (Hussein and Brasel 2001). Only a few studies have analyzed the interaction between microbial spores, or the effect of co-exposure to mycotoxins and microbes.

2.3.1 In vitro studies on interactions of mycotoxins

Co-exposure to two or more mycotoxins has been studied in vitro with several different cell types. Most of the studies have found a synergistic effect either on toxicity (Bacon et al. 1995), growth/DNA synthesis inhibition (Creppy et al. 1980, Jones et al. 1995, Groten et al. 1998) or superinduction of inflammatory mediators together with mitogens (Shahan et al. 1994, Rotter and Oh 1996, Wong et al. 1998). However, there are several studies which report a transient response, with the effects of the co-exposure ranging from antagonistic to synergistic depending on the dosage and timing of the exposure (Cooray et al. 1982, Koshinsky et al. 1992, Kurz and Czuprynski 1992, Madhyastha et al. 1994, Ji et al. 1998, Thuvander et al. 1999, Tajima et al. 2002). This seems to be especially true for the trichothecenes such as T-2 toxin, deoxynivalenol (DON), and nivalenol (NIV). Some studies e.g. on co-exposure to citrinin, ochratoxin and/or aflatoxin have found only additive or even antagonistic responses (Vesela et al. 1983, Blaude et al. 1990, Braunberg et al. 1994, Edrington et al. 1995, Rossano et al. 1999). The in vitro studies of interactions between mycotoxins and/or microbial components are summarized in Table 2.

Mycotoxin exposure may also enhance the allergic response, as the co-exposure to toxin-producing fungi Trichoderma viride together with anti IgE antibody was shown to potentiate histamine release (Larsen 1996). Furthermore, bacterial components and fungal spores enhance the release of histamine both in antigen stimulation of human leukocytes (IgE mediated reaction) and in mediator release triggered by nonimmunological reactions. These nonimmunological reactions were suggested to be mediated by the bacterial peptidoglycans through an interaction with lectins (Norm 1994).
Table 2. Summary of \textit{in vitro} interaction studies on mycotoxins and/or microbial components.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Exposure</th>
<th>Main finding</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Synergistic interaction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatoma tissue culture</td>
<td>Citrinin + ochratoxin A</td>
<td>RNA, DNA synthesis inhibition ↑↑</td>
<td>Creppy et al. 1980</td>
</tr>
<tr>
<td>Guinea pig BAL cells</td>
<td>LPS + fungal spores</td>
<td>Superoxide anion production ↑↑</td>
<td>Shahen et al. 1994</td>
</tr>
<tr>
<td>Chicken embryos</td>
<td>FB, fusaric acid</td>
<td>Toxicity ↑↑</td>
<td>Bacon et al. 1995</td>
</tr>
<tr>
<td>Yeast cells</td>
<td>T-2 + verrucarin A</td>
<td>Growth inhibition ↑↑</td>
<td>Jones et al. 1995</td>
</tr>
<tr>
<td>Mouse macrophages</td>
<td>LPS + FB1</td>
<td>NO production ↑↑</td>
<td>Rotter &amp; Oh 1996</td>
</tr>
<tr>
<td>Mouse fibroblasts</td>
<td>T-2 + DON + NIV + ZEA + FB-1</td>
<td>DNA synthesis inhibition +↑↑</td>
<td>Groten et al. 1998</td>
</tr>
<tr>
<td>Mouse macrophage</td>
<td>LPS + DON</td>
<td>IL-6 production, IL-1β expression ↑↑</td>
<td>Wong et al. 1998</td>
</tr>
<tr>
<td><strong>Transient response</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human PBL</td>
<td>Patulin + cysteine</td>
<td>SCE frequency ↑↑, DNA synthesis inhibition ↓↓</td>
<td>Cooray et al. 1982</td>
</tr>
<tr>
<td>Bovine monocytes</td>
<td>LPS + AFB1</td>
<td>IL-1 production: high dose ↓↓, low dose ±</td>
<td>Kurtz and Czuprynski 1992</td>
</tr>
<tr>
<td>Mouse macrophages</td>
<td>LPS + DON</td>
<td>TNF-α, IL-6 production ↑↑, NO production ↓↓, H2O2 production: high dose ↓↓, low dose ↑↑</td>
<td>Ji et al. 1998</td>
</tr>
<tr>
<td>Mouse fibroblasts</td>
<td>T-2 + DON + NIV + ZEA + FB-1</td>
<td>DNA synthesis inhibition: high dose ↓↓, low dose ↑↑</td>
<td>Tajim et al. 2002</td>
</tr>
<tr>
<td><strong>No interaction or antagonistic response</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken embryos</td>
<td>Citrinin or Ochratoxin A</td>
<td>Toxicity +</td>
<td>Vesela et al. 1983</td>
</tr>
<tr>
<td>Rat and human PBL</td>
<td>DON, acetyldON, ZEA</td>
<td>Proliferation ±</td>
<td>Atkinson and Miller 1984</td>
</tr>
<tr>
<td>Rat spleen lymphocytes, vero cells</td>
<td>19 triphenylcarbazoles</td>
<td>Protein synthesis inhibition +/−</td>
<td>Thompson and Wannemacher 1986</td>
</tr>
<tr>
<td>Bacteria</td>
<td>AFB1 + CPA</td>
<td>Genotoxicity ±</td>
<td>Yates et al. 1987</td>
</tr>
<tr>
<td>Rat hepatocytes</td>
<td>AFB1 + oxysteroycline</td>
<td>RNA, protein synthesis ±, glucogen accumulation ↓↓</td>
<td>Blaude et al. 1990</td>
</tr>
<tr>
<td>Chicken embryos</td>
<td>DON, 15-ADON, HT-2</td>
<td>Toxicity +</td>
<td>Rotter et al. 1991</td>
</tr>
<tr>
<td>Swine renal cortical cubes</td>
<td>Citrinin + ochratoxin A</td>
<td>transport, protein synthesis ±</td>
<td>Braunberg et al. 1994</td>
</tr>
<tr>
<td>Chicken embryos</td>
<td>AFB1 + ochratoxin A</td>
<td>Toxicity ±</td>
<td>Edgrinton et al. 1995</td>
</tr>
<tr>
<td>Human monocytes</td>
<td>LPS + AFB1</td>
<td>IL-1α, IL-6 and TNF-α production ↓↓</td>
<td>Rossano et al. 1999</td>
</tr>
<tr>
<td>Chicken embryos</td>
<td>FB + FB2 + FB3</td>
<td>Toxicity +</td>
<td>Henry and Wyatt 2001</td>
</tr>
</tbody>
</table>

↑↑ = synergistic effect, ↓↓ = antagonistic effect + = additive effect ± = no effect, LPS = lipopolysaccharide, PBL = peripheral blood leukocytes, BAL = bronchoalveolar lavage, NIV = nivalenol, AFB1 = aflatoxin B1, ZEA = zearalenone, DON = deoxynivalenol, FB1 = fusarinisin B1, CPA = cyclopiazonic acid, DAS = diacetoxyscirpenol, 15-ADON = 15-acetyldeoxynivalenol
2.3.2 In vivo studies on interactions of mycotoxins

The majority of the in vivo studies on interactions of the mycotoxins have used poultry or swine as the test animal, since the interest has been the sensitivity of the farm animals to contaminated feed. There are only a few studies about the inhalation exposure to several toxins simultaneously, although there is evidence that inhalation is more than 100 times more effective a route of exposure compared to the oral exposure (Creasia et al. 1990). Most of the combinations tested in feeding studies with poultry and swine had only additive or less than additive effect on toxicity (Kubena et al. 1997, Harvey et al. 1994), although conflicting reports can be found even for the same combinations (Kubena et al. 1989, Harvey et al. 1996).

Inhalation is assumed to be the main route of exposure in a moldy house, thus it is interesting that the few studies done using inhalation reveal a synergistic effect in co-exposure of guinea-pigs to the microbial component β(1-3)-D-glucan together with LPS (Fogelmark et al. 1994, 2001). In oral exposure, the trichotheccenes DON and T-2 are able to enhance the inflammatory responses to endotoxin LPS in mice (Tai and Pestka 1988b, Taylor et al. 1991, Zhou et al. 1999, Zhou et al. 2000, Islam et al. 2002). A similar interaction is the increased hepatotoxicity of aflatoxin B by exposure to LPS (Barton et al. 2000a, 2000b, 2001, Luyendyk et al. 2002). Furthermore, co-exposure to the mycotoxins like aflatoxin, ochratoxin and trichotheccenes increases their acute toxicity to mice (Lindenfelser et al. 1974, Shepherd et al. 1981), caterpillars (Dowd et al. 1989) and guinea-pigs (Pier et al. 1989).

2.3.3 Effect of mycotoxins on immunoresponse

One of the typical adverse health effects associated with exposure to a moisture damaged environment is an increase in airway-infections and flu-like symptoms. Interestingly, some of the mycotoxins, especially trichotheccenes, have a profound effect on host-defense after bacterial or viral infection. The ability of a mycotoxin to modulate immunoresponses in viral infection has been noted by Vieta (1996), who detected in vitro a marked antimicrobial effect against Candida albicans and HIV-1 after exposure to mycotoxin citreoviridin. In contrast, the microbial components LPS, lipotheichoic acid (LTA) and LAM have been shown to increase the susceptibility of macrophages against HIV-1 (Moriuchi et al. 1998)
In animal models, the exposure to trichothecene T-2 toxin has been shown to compromise immunoresistance against infections caused by a variety of infective agents such as mycobacteria, staphylococci, listeria, aspergillus, salmonella, and toxoplasma (Kanai and Kondo 1984, Yarom et al. 1984, Corrier and Ziprin 1987, Niyo et al. 1988, Tai and Pestka 1988, 1990, Ziprin and Elissalde 1990, Venturini et al. 1996). Similar effects have been detected with diacetoxyscirpenol (DAS) (Ziprin and Corrier 1987), ZEA (Pestka et al. 1987), ochratoxin A (Elissalde et al. 1994, Muller et al. 1995), gliotoxin (Sutton et al. 1996), aflatoxin (Awadalla 1998) and fumonisins (Li et al. 2000).

However, mycotoxin exposure may also enhance the resistance to infection (Corrier and Ziprin 1986, Esoula et al. 1988, Cooray and Jonsson 1990, Atroshi et al. 1994, Dresden Osborne et al. 2002), or the response can change from immunosuppression to immunostimulation depending on the agent and the timing of the exposure (Ziprin et al. 1987, Corrier et al. 1987, Corrier and Wagner 1988, Ziprin and McMurray 1988, Bottex et al. 1990, Bekesi et al. 1997). It seems likely that the different outcomes of the mycotoxin exposure in experimental studies are the result of the different exposure parameters, since many factors which can influence the host resistance are not known. The in vivo studies of the effect of mycotoxins on host resistance are summarized in Table 3.
Table 3. Summary of the *in vivo* studies on the effects of mycotoxins on host resistance.

<table>
<thead>
<tr>
<th>Test animal (exposure route)</th>
<th>Mycotoxin</th>
<th>Infective agent</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Immunostimulation, enhanced host resistance</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse (oral)</td>
<td>T-2</td>
<td><em>Listeria monocytogenes</em></td>
<td>Corrier and Ziprin 1986</td>
</tr>
<tr>
<td>Mouse (oral)</td>
<td>Patulin</td>
<td><em>Candida albicans</em></td>
<td>Escoula et al. 1988</td>
</tr>
<tr>
<td>Mouse (intragastric)</td>
<td>T-2</td>
<td><em>Escherichia coli, Staphylococcus aureus</em></td>
<td>Cooray and Jonsson 1990</td>
</tr>
<tr>
<td>Mouse (oral)</td>
<td>T-2, DON</td>
<td><em>Mycobacterium avium, Staphylococcus pyogenicus</em></td>
<td>Atroshi et al. 1994</td>
</tr>
<tr>
<td>Mouse (oral)</td>
<td>Fumonisins</td>
<td><em>Trypanosoma cruzi</em></td>
<td>Dresden Osborne et al. 2002</td>
</tr>
<tr>
<td><strong>Transient response depending on the exposure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse (intragastric)</td>
<td>T-2</td>
<td><em>Listeria monocytogenes, Salmonella typhimurium</em></td>
<td>Ziprin et al. 1987</td>
</tr>
<tr>
<td>Mouse (intragastric)</td>
<td>T-2</td>
<td><em>Listeria monocytogenes</em></td>
<td>Corrier et al. 1987</td>
</tr>
<tr>
<td>Mouse (intragastric)</td>
<td>T-2</td>
<td><em>Babesia microti</em></td>
<td>Corrier and Wagner 1988</td>
</tr>
<tr>
<td>Mouse (intragastric)</td>
<td>T-2</td>
<td><em>Listeria monocytogenes, Mycobacterium bovis, Salmonella typhimurium</em></td>
<td>Ziprin and McMurray 1988</td>
</tr>
<tr>
<td>Mouse (intraperitoneal)</td>
<td>DAS</td>
<td><em>Salmonella typhimurium</em></td>
<td>Bottex et al. 1990</td>
</tr>
<tr>
<td>Chicken (oral)</td>
<td>F2, T-2</td>
<td><em>Cryptosporidium baileyi</em></td>
<td>Bekesi et al. 1997</td>
</tr>
<tr>
<td><strong>Immunosuppression, reduced host resistance</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse (oral)</td>
<td>T-2</td>
<td><em>Mycobacterium bovis, Mycobacterium tuberculosis</em></td>
<td>Kanai and Kondo 1984</td>
</tr>
<tr>
<td>Rat (intraperitoneal)</td>
<td>T-2</td>
<td><em>Staphylococcus aureus</em></td>
<td>Yarom et al. 1984</td>
</tr>
<tr>
<td>Mouse (oral)</td>
<td>T-2</td>
<td><em>Listeria monocytogenes</em></td>
<td>Corrier and Ziprin 1987</td>
</tr>
<tr>
<td>Mouse (oral)</td>
<td>DON, ZEA</td>
<td><em>Listeria monocytogenes</em></td>
<td>Pestka et al. 1987</td>
</tr>
<tr>
<td>Mouse (intragastric)</td>
<td>DAS</td>
<td><em>Listeria monocytogenes</em></td>
<td>Ziprin and Corrier 1987</td>
</tr>
<tr>
<td>Rabbit (oral)</td>
<td>T-2</td>
<td><em>Aspergillus fumigatus</em></td>
<td>Niyo et al. 1988</td>
</tr>
<tr>
<td>Mouse (oral)</td>
<td>T-2</td>
<td><em>Salmonella typhimurium</em></td>
<td>Tai and Pestka 1988</td>
</tr>
<tr>
<td>Mouse (oral)</td>
<td>T-2</td>
<td><em>Salmonella typhimurium</em></td>
<td>Tai and Pestka 1990</td>
</tr>
<tr>
<td>Chicken (oral)</td>
<td>T-2</td>
<td><em>Salmonella typhimurium</em></td>
<td>Ziprin and Elissalde 1990</td>
</tr>
<tr>
<td>Chicken (intraperitoneal)</td>
<td>Ochratoxin A</td>
<td><em>Salmonella typhimurium</em></td>
<td>Elissalde et al. 1994</td>
</tr>
<tr>
<td>Mouse (intraperitoneal)</td>
<td>Ochratoxin A</td>
<td><em>Escherichia coli, Pasteurella multocida</em></td>
<td>Muller et al. 1995</td>
</tr>
<tr>
<td>Mouse (intragastric)</td>
<td>T-2, AFB1</td>
<td><em>Toxoplasma gondii</em></td>
<td>Venturini et al. 1996</td>
</tr>
<tr>
<td>Mouse (intraperitoneal)</td>
<td>Gliotoxin</td>
<td><em>Aspergillus fumigatus</em></td>
<td>Sutton et al. 1996</td>
</tr>
<tr>
<td>Quail-chicks (oral)</td>
<td>AFB1</td>
<td><em>Eimeria</em></td>
<td>Awadalla 1998</td>
</tr>
<tr>
<td>Chicken (oral)</td>
<td>FB1, moniliformin</td>
<td><em>Escherichia coli</em></td>
<td>Li et al. 2000</td>
</tr>
</tbody>
</table>

AFB1 = aflatoxin B1, ZEA = zearalenone, DON = deoxynivalenol, FB1 = fumonisin B1, DAS = diacetoxyisocirpenol, T-2 = T-2 toxin
2.4. Inflammation

Inflammation is a local response of tissues to injury or irritation characterized by capillary dilatation, leukocytic infiltration, pain, swelling, redness and heat. Inflammatory reactions occur in the affected blood vessels and adjacent tissues in response to an injury or abnormal stimulation caused by a physical, chemical, or biologic agent, serving as a mechanism initiating the elimination of noxious agents and of damaged tissue.

2.4.1 Cells involved in inflammation

The immune system is a complex network of inflammatory cells and their interactions. The role of macrophages and epithelial cells in inflammation are described in the following short overview more thoroughly, since they are utilized as a cell culture in this study.

*Epithelial cells*

One of the first cell types that an inhaled particle will encounter is the epithelial cell lining the alveolar space in lungs. These cells are capable of producing inflammatory mediators such as cytokines IL-1, IL-6 and chemotactic IL-8, thus activating the innate host defense by recruiting macrophages and neutrophils into the battle against the invading microbes (Stadnyk 1994). Several airway disorders such as asthma are associated with increased levels of inflammatory cytokines in bronchial epithelial cells, and the production of these agents is upregulated by the proinflammatory cytokines IL-1 and TNF-α (Takizawa 1998). Exposure to organic dust collected from the indoor environment induces the production of cytokines in epithelial cells, and this capacity has been utilized to compare indoor air environments according to the inflammatory potential of dust samples (Allerman et al. 2000).

*Macrophages*

Macrophages are phagocytic cells derived from the monocytes in the bloodstream. They are “the garbage man” of the immune system; they recognize foreign material, and destroy it by phagocytosis and production of microbicidal products such as nitric oxide (NO) and reactive oxygen species (ROS), hydrolytic enzymes, bioactive lipids and several cytokines such as TNF-α, IL-1 and IL-6. Macrophages also present the antigens of the phagocytized microbes.
via their major histocompatibility complex (MHC) molecules, which activates other inflammatory cells to eliminate the foreign particle (Delves and Roitt 2000). In the lungs, macrophages are present in alveolar surfactant film (alveolar macrophages), lung connective tissue (interstitial macrophages) and in the capillaries (intravascular macrophages) (Lohmann-Matthes et al. 1994).

**Dendritic cells**

Antigen presentation is also the function of dendritic cells, which seek the help of lymphocytes from the local lymph node after encountering foreign substances. For successful activation of T-lymphocytes, antigen presenting cells must present additional costimulatory signals (such as CD80 or CD86) together with the peptide-MHC complex, concurrently guaranteeing that the adaptive immune response is directed against nonself-antigens (Pulendran et al. 2001).

**Granulocytes**

Mediators produced by macrophages attract neutrophilic granulocytes to the site of inflammation. This leads to increased phagocytosis of the particles and production of numerous microbicidal products such as proteases, lipids and cytokines. Neutrophils are considered to be the major cellular component in the defense against microbes, since patients with marked neutropenia often have impaired resistance against bacterial and fungal infections. However, extensive and sustained production of inflammatory mediators may lead to adverse effects on local cells and tissues (Sibille and Marchandise 1993). Mast cells, eosinophils and basophils are bone marrow-derived granulocytes which are important effector cells in IgE mediated adaptive immunity, and in the pathophysiology of allergic diseases (Delves and Roitt 2000).

**Lymphocytes**

Lymphocytes e.g. T- and B-cells are the effector cells of the adaptive host defense and thus have the ability to respond faster to previously encountered antigen. T-cells participate both in development of cell mediated immune responses and in the activation of antibody production by B-lymphocytes, as well as direct elimination of infected cells. B-lymphocytes are
responsible for the production of immunoglobulins, the antigen-specific antibodies, which in turn activate and target other functions of the host defense system such as opsonization, complement activation and antibody-dependent cytotoxicity (Moore et al. 2001). A subgroup of lymphocytes called natural killer (NK) cells can effectively activate macrophages by producing INF-γ and kill infected and malignant cells directly by lysis (Delves and Roitt 2000).

2.4.2 Mediators of inflammation

The inflammatory reaction is mediated by a complex assembly of diverse agents, including amines, lipids, peptides, free radicals, proteases and cytokines (Barnes 1998). The free radicals nitric oxide (NO) and reactive oxygen species (ROS), along with the cytokines interleukin 1 (IL-1), tumor necrosis factor α (TNF α), interleukin 6 (IL-6) and interleukin 10 (IL-10) are discussed below in more detail, as they are the inflammatory mediators measured in this study.

Nitric oxide

NO is a mediator in many biological processes, acting as a paracrine messenger, vasodilator and source of microbicidal oxidants. This free radical is formed in several cell types by the conversion of L-arginine to L-citrulline by either the constitutive or the inducible form of the enzyme NO-synthase, the inducible form being the main source of NO in inflammatory reactions (Singh and Evans 1997). The produced NO is transformed rapidly to nitrite and nitrate, which may be measured as a marker for NO production. Together with superoxide anions, NO forms the highly reactive compound, peroxynitrite, which is thought to be involved in the hyperresponsiveness of the asthmatic airways (Barnes et al. 1998). Elevated production of NO and the expression of inducible NO-synthase (iNOS) are commonly associated with inflammatory diseases of the respiratory tract, presumably via the effect of toxic derivatives such as peroxynitrite (van der Vliet et al. 2000).

Reactive oxygen species

Reactive oxygen species (ROS) such as superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂) and the hydroxyl radical (OH⁻) are important in both intracellular and extracellular defense
against micro-organisms. ROS are produced by a multicomponent enzyme NADPH oxidase, further processed by superoxide dismutases (SODs), and they are released during the respiratory burst in response to various stimuli. In addition to macrophages and neutrophils, also other cell types such as endothelial cells are capable of producing O₂ metabolites, although the levels are lower when they are formed by enzyme-linked processes other than NADPH oxidase system (Lohmann-Matthes et al. 1994). Due to their oxidizing capacity, ROS may provoke tissue injury on account of their cytotoxicity, but normally the production of oxidants is compensated by antioxidant mechanisms in the respiratory tract (Barnes et al. 1998). Exposure to fungal spores has been shown to induce increased production of ROS in vitro (Ruotsalainen et al. 1998, Shahan et al. 2000).

**Cytokines**

Cytokines are soluble mediators involved in the regulation of immune responses, where they contribute to the recognition of pathogens, recruitment of neutrophils and macrophages and removal of the invading microbe. The interactions and overlapping of these functions form a network, where the produced cytokine may act as a feedback signal or substitute for another cytokine.

Among the proinflammatory cytokines, IL-1 and TNF-α are considered as the cytokines of the early response to the inflammatory stimuli. IL-1 is a cytokine which exists in two forms, α and β. These have similar properties and bind to the same receptor. Nevertheless, they are coded by different genes and importantly, IL-1α is usually found intracellularly whereas IL-1β is released to the extracellular space. IL-1 is pyrogenic and it induces the release of neutrophils, the growth of thymocytes and the production of other cytokines (Kelley 1990, Barnes et al. 1998). TNF has also α and β forms, which are produced mainly by macrophages, T lymphocytes, mast cells and epithelial cells (TNF-α) and activated lymphocytes (TNF-β). TNF-α enhances phagocytosis, the respiratory burst and the release of proteases, the expression of adhesion molecules and production of other cytokines. It is also directly cytotoxic, capable of inducing both apoptosis and necrosis (Barnes et al. 1998, Luster et al. 1999).
IL-6 has multiple biologic functions including the induction of fever, the production of acute phase proteins, growth and differentiation of T and B lymphocytes, thus contributing to the initiation and extension of the inflammatory process. IL-6 may also have anti-inflammatory effects, since it can inhibit the production of other proinflammatory cytokines. Asthmatic individuals have increased basal release of this cytokine, and elevated levels in serum are associated with bacterial infections such as pneumonia (Van Snick 1990, Barnes et al. 1998).

Anti-inflammatory IL-10 is an example of a cytokine that inhibits both the innate and adaptive immune systems. This cytokine is produced constitutively in healthy lungs, but the expression is decreased in the lungs of asthmatic individuals. IL-10 is a strong inhibitor of macrophage function including suppression of the cytokine production, receptor expression and synthesis of ROS and NO. On the other hand, there is an indication that the recruitment of phagocytes and the growth of B-lymphocytes and mast cells can be enhanced by this cytokine (Barnes et al. 1998, Mehrad and Standiford 1999).

Chemokines are a small family of cytokines, consisting of structurally similar molecules like IL-8, MCP-1, MIP-1β, and RANTES. In addition to phagocytes, also endothelial cells, keratinocytes, fibroblasts and smooth muscle cells can synthesize these small polypeptides. Chemokines function as an alarm system for phagocytic cells, inducing the migration of the macrophages and lymphocytes to the inflammatory site. Infection or physical damage to tissues induces the production of chemokines, which bind to proteoglycan molecules in the extracellular matrix and on endothelial cell surfaces. Consequently, the migration of leukocytes is directed by the increasing gradient of matrix-associated chemokine molecules (Janeway and Travers 1997).

**Cell adhesion molecules**

The binding of the leukocytes to each other, to endothelial cells, or to extracellular matrix is mediated by cell adhesion molecules (CAMs), which can be regarded as insoluble mediators of inflammation. Adhesion molecules are cell-surface proteins, which include members of the immunoglobulin superfamily, integrins, selectins, mucins and cadherins. Expression and activation of adhesion molecules are induced by cytokines (Lodish et al. 2000).
The different mediators of inflammation co-operate in their attempts to contain and remove the noxious agent. For example, the extravasation of leukocytes from bloodstream to the inflamed tissue is orchestrated by several cytokines and CAMs. First, the inflammatory signal, e.g. TNF-α, induces the expression of P-selectin in endothelial cells. Leukocytes circulating in the bloodstream bind weakly to P-selectin, and thus are slowed down, "rolling" along the endothelium. Next, the adhesion of the leukocyte to the endothelial cell is tightened by cytokine-activated integrins, leading to the extravasation of the leukocytes through the epithelium. Finally, the extravasated leukocytes continue their migration towards the inflammatory site, guided by the gradient of the produced chemokines (Janeway and Travers 1997).

2.4.3 Receptors associated with microbe recognition

The function of the immune system is dependent on the ability of the effector cells to recognize infectious microbes and discriminate between foreign (nonself) and own (self) antigens. The cells of the adaptive immune system handle this task by expressing a wide variety of randomly generated antigen receptors. Subsequently, the cells reacting to self antigens are eliminated to establish a group of effector cells with the highest possible diversity without autoreactivity. However, the full response of the antigen-specific cells requires several days and thus it is the responsibility of the innate immune system to provide a faster means to contain the infection, and direct the adaptive host defense against invading microbes (Medzhitov and Janeway 2000).

Phagocytosis is a key factor in the containment and removal of infective agents. Phagocytic cells in general possess a wide variety of membrane receptors. Opsonized particles activate phagocytosis via Fc-, complement- and lectin receptors. Through Fc–receptors, macrophages are able to handle all antibody-bound antigens, whereas complement receptors recognize antigens opsonized by components of complement and β-glucan. The lectin –binding receptors interact with lectins on micro-organisms and plant cells (Lohmann-Matthes et al. 1994).

The cells of the innate immune system recognize a number of conserved microbial features, termed as pathogen-associated molecular patterns (PAMPs), and this is accomplished via their pattern-recognition receptors (PRRs). These receptors can be divided into three classes
according to their function: secreted, endocytic and signaling receptors. Secreted PRRs, e.g. mannan-binding lectin binds to the microbial cell wall, thus tagging them to be recognized by phagocytes and the complement system (Medzhitov and Janeway 2000).

Endocytic PRRs can be found on the surface of phagocytes, mediating the uptake and consequent breakdown of the microbe in the lysosomes. This enables the peptides of the infective agent to be presented on the surface of the macrophage in order to further activate T-cells. Example of these endocytic receptors are macrophage scavenger receptors such as macrophage receptor with a collagenous structure (MARCO). These receptors are rapidly induced in most tissue macrophages by treatment with bacteria or LPS in vitro, although noninfectious stimuli such as particles can also upregulate their expression (Peiser et al. 2002).

Signaling PRRs activate signal-transduction pathways, leading to the expression of various genes involved in inflammatory responses. These include toll-like receptors (TLRs), which are transmembrane proteins expressed on dendritic cells and macrophages. In humans, there are currently ten known TLRs which are activated by a diverse array of bacterial and viral products including mycobacterial lipopeptides, peptidoglycan from gram-positive bacteria, zymosan from yeast, and lipopolysaccharide from gram-negative bacteria (Barton and Medzhitov 2002, O'Neill 2002). Interestingly, also sequences of bacterial DNA can be recognized through toll-like receptors, despite their similarities with human DNA (Bauer and Wagner 2002).

2.4.4 Signal transduction

Inflammatory proteins including enzymes, protein and peptide mediators responsible for stimulation of innate immune system are not constitutively expressed but the production must be induced by the microbial antigen. The signal is delivered from the receptor to the nucleus through a signal-transduction pathway; a cascade of enzymes ultimately activating the transcription of the relevant inflammatory mediators. The TLRs have several signal transduction pathways which activate the appropriate effector mechanisms. These include the proapoptotic caspase cascades, Jun N-terminal kinase (JNK)/activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) inducing pathways (Silverman and Maniatis 2001).
The expression of inflammatory mediators is ultimately regulated by transcription factors such as the universal transcription factors NF-κB and AP-1. These bind to consensus sequences in the promoter regions of several genes involved in inflammation, whereas other transcription factors e.g. nuclear factor of activated T-cells (NF-AT) have a more limited set of target genes (Barnes et al. 1998). In unstimulated cells, the NF-κB protein family, which consists of five members (Rel A/p65, p50, p52, c-Rel and Rel-B), is located in the cytoplasm associated with the IκB family of inhibitory proteins, or as large precursor proteins p100 and p105. Signal transduction pathways activated by extracellular signals like PAMPs lead to destruction of IκB, which frees the NF-κB to be translocated to the nucleus and there to bind to the promoter regions of their target genes (Ghosh et al. 1998, Silverman and Maniatis 2001). The binding of lipopolysaccharide to the toll-like receptor -4 is depicted as an example of signal transduction in Figure 1.
**Figure 1.** The PAMP interacts with TLR to activate signal transduction pathway leading to transcription of target genes. For example, LPS binds to the complex formed by TLR4 and co-receptors CD14 and MD2, which then recruits an adapter molecule MyD88 to the cytoplasmic Toll/IL-1 receptor (TIR)-domain, leading to further recruitment of IL-1R associated kinase (IRAK). Subsequently, IRAK activates an adapter molecule TNF receptor associated factor 6 (TRAF6), which becomes ubiquitinated and activates the TAK1/TAB1/TAB2 complex. TAK1 then activates the IκB kinase complex (IKK), which phosphorylates IκB designating it to degradation by the proteasome. The resulting free NF-κB then translocates to the nucleus and binds to consensus sequences in the promoter regions of the genes involved in inflammation (modified from Silverman and Maniatis 2001).
3. THE AIMS OF THE STUDY

The overall aim of the current study was to characterize the potency of different indoor air microbes to induce the production of inflammatory markers or cytotoxicity in vitro. The detailed aims were as follows:

1. To study the ability of different isolates of certain species of environmental mycobacteria to cause cytotoxicity and trigger the production of inflammatory mediators in mouse macrophages. (I)

2. To study whether human cell lines respond to the same stimuli as mouse cells, and to evaluate the representativeness of the studied markers and the time scale of the induced responses. (II)

3. To compare the cytotoxic and inflammatory potential of indoor air fungi and bacteria. (III)

4. To study the relation between the biological activity and the produced metabolite profile of fungal strains. (IV)

5. To study the interactions of selected microbes in co-exposure. (V)
4. MATERIALS AND METHODS

4.1 Exposure agents

4.1.1 Microbes (I-V)

A group of mycobacterial isolates (studies I and II) were selected to study the differences between potentially pathogenic and apathogenic species, and to compare different strains within the same species. For the further studies (III-V), bacterial and fungal species were selected, each to represent a group of microbes: *Streptomyces californicus*, a gram-positive bacterium as an indicator of moisture and mould damage; *Pseudomonas fluorescens*, a gram negative bacterium as a species typical for outdoor air; *Bacillus cereus*, a gram-positive bacterium as a typical species in indoor air; *Stachybotrys chartarum*, a toxic fungi as an indicator of moisture and mould damage; *Aspergillus versicolor*, a common fungus in indoor air and an indicator of moisture and mould damage; and *Penicillium spinulosum*, a common fungus in indoor air.

Isolation and cultivation of the microbes were done in the Laboratory of Microbiology, National Public Health Institute, Kuopio and identification of the strains was confirmed in international laboratories as summarized in Table 4.

4.1.2 Fungal metabolites (IV, V)

The metabolites of *Stachybotrys* ssp. were tested to compare the biological activity of the strains and the produced metabolite profiles (study IV). For the interaction studies, metabolites were selected to represent the most common mycotoxins produced by moldy house microbes (sterigmatocystin, ochratoxin, citrinin), metabolites of *Stachybotrys* (satratoxins, verrucarin, atranones, trichodermin) and some relative compounds (7-α-hydroxytrichodermol, SMTP-7, staplabin) (Study V).

Isolation and identification of the metabolites from the methanol extracts of fungal cultures was done by the Mycology Group, Biocentrum-DTU, Technical University of Denmark as summarized in Table 5.
Table 4. Summary of the microbes used in the studies, their origin, identification and culture conditions.

<table>
<thead>
<tr>
<th>Microbe</th>
<th>Origin and isolation</th>
<th>Culture conditions</th>
<th>Identification</th>
<th>study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycobacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mycobacterium avium</em>-complex (strains BA12, BA31)</td>
<td>indoor air of a mouldy building during demolition work</td>
<td>Mycobacteria 711-agar with OADC-enrichment at 30°C for 5 weeks</td>
<td>Laboratory of Microbiology, KTL, Kuopio, Finland</td>
<td>II</td>
</tr>
<tr>
<td><em>Mycobacterium scrofulaceum</em> (strain BA24)</td>
<td>indoor air of a mouldy building during demolition work</td>
<td>Mycobacteria 711-agar with OADC-enrichment at 30°C for 5 weeks</td>
<td>Laboratory of Microbiology, KTL, Kuopio, Finland</td>
<td>I</td>
</tr>
<tr>
<td><em>Mycobacterium terrae</em> (strains BA20, BA26)</td>
<td>indoor air of a mouldy building during demolition work</td>
<td>Mycobacteria 711-agar with OADC-enrichment at 30°C for 5 weeks</td>
<td>Laboratory of Microbiology, KTL, Kuopio, Finland</td>
<td>II, V</td>
</tr>
<tr>
<td>Bacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus cereus</em> (strain B64)</td>
<td>indoor air of a residence using a six-stage impactor</td>
<td>Tryptone yeast glucose agar (TYG) at 20°C for 5 days.</td>
<td>National Veterinary and Food Research Institute, Kuopio Regional Laboratory, Finland.</td>
<td>III, V</td>
</tr>
<tr>
<td><em>Streptomyces californicus</em> (strain A4)</td>
<td>indoor air of moisture problem building using a six-stage impactor</td>
<td>Tryptone yeast glucose agar (TYG) at 20°C for 5 days.</td>
<td>DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany</td>
<td>III, V</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em> (strain N78)</td>
<td>indoor air of a residence using a six-stage impactor</td>
<td>Tryptone yeast glucose agar (TYG) at 20°C for 5 days.</td>
<td>National Veterinary and Food Research Institute, Kuopio Regional Laboratory, Finland.</td>
<td>III, V</td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus versicolor</em> (strain HT486)</td>
<td>indoor air of moisture problem building using a six-stage impactor</td>
<td>2% malt extract agar (MEA) at 25°C for 7 days.</td>
<td>Centraal Bureau of Schimmelcultures, Utrecht, the Netherlands</td>
<td>III, V</td>
</tr>
<tr>
<td><em>Penicillium spinulosum</em> (strain HT581)</td>
<td>indoor air of moisture problem building using a six-stage impactor</td>
<td>2% malt extract agar (MEA) at 25°C for 7 days.</td>
<td>Centraal Bureau of Schimmelcultures, Utrecht, the Netherlands</td>
<td>III, V</td>
</tr>
<tr>
<td><em>Stachybotrys chartarum</em> (strain HT 580)</td>
<td>damaged building material sample</td>
<td>2% malt extract agar (MEA) at 25°C for 7 days.</td>
<td>Centraal Bureau of Schimmelcultures, Utrecht, the Netherlands</td>
<td>III, V</td>
</tr>
<tr>
<td><em>Stachybotrys chartarum</em> (20 strains*)</td>
<td>damaged building material sample</td>
<td>potato sucrose agar (PSA) at 25°C for 7 days.</td>
<td>Biozentrum-DTU, Technical University of Denmark, Denmark</td>
<td>IV</td>
</tr>
</tbody>
</table>

*For details see the original study Ruotsalainen et al. 1998*
Table 5. Summary of the fungal metabolites used in the studies, their origin, detection and producing microbes.

<table>
<thead>
<tr>
<th>Metabolites of Stachybotrys</th>
<th>Metabolite</th>
<th>Origin</th>
<th>Detection</th>
<th>Produced by</th>
<th>study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso-satratoxin F</td>
<td>BB Jarvis, University of Maryland, USA</td>
<td>LC-DAD + GC-MS/MS**</td>
<td><em>Stachybotrys chartarum</em>¹¹</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td>Satratoxin G</td>
<td>BB Jarvis, University of Maryland, USA</td>
<td>LC - DAD - MS**</td>
<td><em>Stachybotrys chartarum</em>³</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>Verrucarin J</td>
<td>BB Jarvis, University of Maryland, USA</td>
<td>LC-DAD + GC-MS/MS**</td>
<td><em>Myrothecium spp., Stachybotrys chartarum</em>²,⁹</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td>Atranone B</td>
<td>BB Jarvis, University of Maryland, USA</td>
<td>LC - DAD - MS</td>
<td><em>Stachybotrys chartarum</em>⁵</td>
<td>IV, V</td>
<td></td>
</tr>
<tr>
<td>Atranone D</td>
<td>BB Jarvis, University of Maryland, USA</td>
<td>LC - DAD</td>
<td><em>Stachybotrys chartarum</em>⁷</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td>Atranone E</td>
<td>BB Jarvis, University of Maryland, USA</td>
<td>LC - DAD - MS</td>
<td><em>Stachybotrys chartarum</em>⁵</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>Trichodermin</td>
<td>Løvens Kemiske Fabrik A/S, Ballerup, Denmark</td>
<td>LC - DAD - MS or GC-MS/MS*</td>
<td><em>Trichoderma spp.</em>, <em>Mononniella spp.</em>, <em>Myrothecium spp.</em>, *Stachybotrys spp.*¹</td>
<td>IV, V</td>
<td></td>
</tr>
<tr>
<td>Relative compounds</td>
<td>7-α-hydroxy-trichodermol</td>
<td>BB Jarvis, University of Maryland, USA</td>
<td>LC - DAD - MS</td>
<td><em>Myrothecium roridum</em>²</td>
<td>V</td>
</tr>
<tr>
<td>SMTP-7</td>
<td>Prof. Keiji Hasumi, Tokyo Noko University, Japan</td>
<td>LC - DAD - MS</td>
<td><em>Stachybotrys microspora</em>⁶</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>Staplabin</td>
<td>Prof. Keiji Hasumi, Tokyo Noko University, Japan</td>
<td>LC - DAD - MS</td>
<td><em>Stachybotrys microspora</em>¹²,¹⁵</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>Mycotoxins</td>
<td>Sterigmatocystin</td>
<td>Sigma, St. Louis, MO, USA</td>
<td>LC - DAD - MS</td>
<td><em>Aspergillus spp.</em>, *Chaetomium spp.*¹⁴</td>
<td>V</td>
</tr>
<tr>
<td>Citrinin</td>
<td>Sigma, St. Louis, MO, USA</td>
<td>LC - DAD - MS</td>
<td><em>Penicillium spp</em></td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td>Sigma, St. Louis, MO, USA</td>
<td>LC - DAD - MS</td>
<td><em>Penicillium spp.</em>, <em>Aspergillus spp.</em></td>
<td>V</td>
<td></td>
</tr>
</tbody>
</table>


LC = liquid chromatography; DAD = photo diode array detector; MS = mass spectrometer; MS/MS = tandem mass spectrometer
** prior to GC-MS/MS they are hydrolysed to verrucarol, thus indicative.
* hydrolysed to trichodermol which is also produced by the fungus, thus these two cannot be differentiated
4.2 Cell cultures

Three cell lines (mouse RAW264.7 macrophages, human A549 lung epithelial cells, human 28SC macrophages) were obtained from American Type Culture Collection, Rockville, MD, USA and cultured in cell line specific culture media at 37°C in 5% CO₂ atmosphere.

4.2.1 RAW264.7 mouse macrophages (studies I - V)

The mouse macrophage cell line RAW264.7 originated from a BALB/c mouse, being established from a tumor induced by Abelson murine leukemia virus. The cells were cultured in RPMI 1640 medium supplemented with 10% of heat inactivated fetal bovine serum (FBS), 1% of l-glutamine and 1% of penicillin-streptomycin. (For details see Study I)

4.2.2 A549 human lung epithelial cells (studies II, III)

The human lung epithelial cell line A549 originated from a human alveolar cell carcinoma and possesses the properties of type II alveolar epithelial cells. The cells were cultured in Ham's F-12K medium supplemented with 10% heat inactivated FBS. The cells were primed with INF-γ (10 ng/ml) for 24 hours in order to enhance the response of the cells. Anti-microbial agents (nystatin and penicillin-streptomycin) were added after the dispensing of the cells (For details see Study II).

4.2.3 28SC human macrophages (studies II, III)

The human 28SC macrophages (hematopoietic cell line) was originally established from human peripheral blood mononuclear cells. The cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% of heat inactivated FBS, 0.05 mM 2-mercaptoethanol, 0.03 mM thymidine and 0.01 mM hypoxanthine. The cells were primed with INF-γ (10 ng/ml) for 24 hours in order to enhance their response. Anti-microbial agents (nystatin and penicillin-streptomycin) were added after the dispensing of the cells (For details see Study II).
4.3 Study design

4.3.1 Exposure

Cells (5x10^5 cells/ml) were dispensed to 6-well plates, 2 ml/well. The cells were allowed to adhere and stabilize for 24 hours and, before exposure, fresh complete medium was added. The human macrophage cells grow in suspension and hence they were exposed without changing the culture medium. After the exposure, the adherent cells were resuspended in the culture medium either by scraping (RAW264.7) or by trypsin-incubation (A549) and the cell suspension was centrifuged (5 min, 8000 rpm) in order to separate the cells from the culture medium. Concentration of nitrite in the culture medium and the viability of the exposed cells were measured immediately after the exposure. The remaining supernatants were stored at -80°C for the analyses of cytokines and the cells were frozen immediately in dry ice and stored at -80°C for Western blot analysis.

4.3.2 Dose-response (I-V) and time course studies (I-III)

In studies I-III, the cell lines were exposed to graded doses of microbes to study the dose-response. The time course of the responses was studied by terminating the exposure to a selected dose at different time points up to 48 hours. In study IV, the metabolites of *Stachybotrys* were dissolved in methanol (MeOH) and diluted further in HBSS. The studied doses were selected in order to define the effective dose for all the studied compounds, ranging between 3 pg/ml - 68 μg/ml. In study V, the interaction between different exposures was studied by exposing the macrophages to different doses of each microbe or the metabolite alone and in combination with a low dose of *Streptomyces californicus* (Table 6).

The control cells in all of the studies were exposed to carrier buffer; pure HBSS in studies I, II, III, V and HBSS/MeOH in studies IV and V. The bacterial lipopolysaccharide (LPS) was used as a positive control in studies II-IV. The dose of the positive control was chosen to be high (10 μg/ml) in order to obtain maximum inflammatory responses from all of the cell lines.
Table 6. Summary of doses and time points used in the studies I-V.

<table>
<thead>
<tr>
<th>Study</th>
<th>Cell lines</th>
<th>Exposure</th>
<th>Dose /ml</th>
<th>Time (hrs)</th>
<th>Test</th>
</tr>
</thead>
</table>
| I     | RAW264.7   | *Mycobacterium avium* BA12, BA31  
        |           | *Mycobacterium scrofulaceum* BA24  
        |           | *Mycobacterium terrae* BA20, BA26  | 5x10², 5x10³, 5x10⁴, 5x10⁵, 5x10⁶ cells  | 24 | Dose-response |
|       |            |          |          | 5x10³ cells | 2, 4, 8, 16, 24, 48 | Time-response |
| II    | RAW264.7   | *Mycobacterium avium* BA12, BA31  
        | 28SC     |           | 10⁷, 10⁸, 10⁹ cells | 24 | Dose-response* |
|       | A549       | *Mycobacterium terrae* BA20, BA26  |          | 10⁷ cells | 3, 6, 12, 24, 48 | Time response |
| III   | RAW264.7   | *Bacillus cereus, Streptomyces californicus,*  
        | 28SC     | *Pseudomonas fluorescens,* *Aspergillus versicolor,*  
        | A549     | *Penicillium spinulosum,* *Stachybotrys chartarum* | 10⁷, 10⁸, 10⁹ spores or cells | 24 | Dose response |
|       |            |          |          | 10⁸ spores or cells | 3, 6, 12, 24, 48 | Time-response |
| IV    | RAW264.7   | iso-satratoxin F  | 680 pg - 68 μg | 24 | Dose-response |
|       |            | verrucarin J  | 3 pg - 66 μg |          | |
|       |            | atranone B  | 560 ng -56 μg |          | |
|       |            | atranone D  | 500 ng - 50 μg |          | |
| V     | RAW264.7   | *Streptomyces californicus* | 10⁷ spores | 24 | Interaction |
|       |            | *Bacillus cereus,* *Pseudomonas fluorescens,*  
        |          | *Mycobacterium terrae,* *Aspergillus versicolor,*  
        |          | *Penicillium spinulosum,* *Stachybotrys chartarum*  | 10⁷, 10⁸, 10⁹ spores or cells | 24 | Dose-response |
|       |            | atranone B, atranone E, sterigmatocystin, citrinin,  
        |          | ochratoxin A | 10, 100, 1000 ng | |
|       |            | satratoxin G  | 1, 10, 100 ng |          | |
|       |            | trichodermin, 7-α-hydroxytrichodermol | 5, 10, 50, 100, 500, 1000 ng | |

*dose response was not done with mouse macrophages (see Study I)*
4.4 Biochemical analyses (I-V)

The concentrations of selected inflammatory markers (cytokines IL-1β, TNF, IL-6 and IL-10; nitrate, reactive oxygen species) were measured from the cell culture medium after the exposure, whereas the viability of the exposed cells, expression of the inducible NO synthase and nuclear binding of NF-κB were analyzed from the cells. The methods and references for used analyses are summarized in Table 7.

Table 7. Measured parameters and applied analyzing method.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Study</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>I-III</td>
<td>Enzyme-linked</td>
<td>Ruotsalainen et al. 1998</td>
</tr>
<tr>
<td>TNF-α</td>
<td>I-V</td>
<td>immunosorbent assay</td>
<td>Gentzyme, R&amp;D systems</td>
</tr>
<tr>
<td>IL-6</td>
<td>I-V</td>
<td>(ELISA)</td>
<td>manufacturer’s protocol</td>
</tr>
<tr>
<td>IL-10</td>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitric oxide (NO)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrate</td>
<td>I-V</td>
<td>Griess</td>
<td>Green et al. 1982</td>
</tr>
<tr>
<td>iNOS</td>
<td>I-III</td>
<td>Western blot</td>
<td>Hirvonen et al. 1996</td>
</tr>
<tr>
<td>ROS</td>
<td>I</td>
<td>Luminometric analysis</td>
<td>Ruotsalainen et al. 1998</td>
</tr>
<tr>
<td>Cytotoxicity</td>
<td>I-V</td>
<td>MTT-test, Trypan blue exclusion</td>
<td>Mosmann 1983</td>
</tr>
</tbody>
</table>

4.5 Statistical analyses

Difference between exposed and control samples were analyzed by using analysis of variance (ANOVA) and a least significant difference (LSD) test. Interactions between microbial exposures were examined using the analysis of variance and Tukey's test (SPSS, version 7.51, SPSS Inc.). The difference was considered to be statistically significant at P<0.05.
5. RESULTS

5.1 Differences between cell lines (Studies II and III)

The concentrations of IL-6 and cytotoxicity after the exposure to three doses of each microbe in three cell lines (combined data from studies I-III) are presented in Table 8. The relative concentrations of cytokines TNF-α, IL-1β and IL-6 in the three cell lines after exposure to one dose of *Streptomyces californicus* are illustrated in figure 2.

5.1.1 Production of inflammatory mediators

Mouse RAW264.7 macrophages were markedly more sensitive to the microbial exposure than human cell lines 28SC and A549 (II). Among the studied inflammatory markers, IL-6 was the most consistent in all different cell types (Figure 2). Regardless of the difference in the sensitivity, basically the same exposure agents induced an increase in IL-6 response in all three cell lines, the exception being human lung epithelial cells which reacted more clearly to *Stachybotrys chartarum*. The greatest variance between the different cell lines was detected in the TNF-α production. The mouse macrophages produced high amounts of TNF-α in contrast to human macrophages which responded only to selected exposures, and human epithelial cells which did not produce TNF-α at all (Figure 2). The NO production was increased somewhat more in human epithelial cells than in the human macrophages. The highest amounts of NO were produced in mouse macrophages (II, III).

5.1.2 Cytotoxicity

Microbial exposure was most toxic to mouse macrophages as both the bacterial and fungal species caused a significant increase in the proportion of dead cells. The bacterial species analyzed were not toxic to human macrophages, and in the human epithelial cell line the number of cells even increased during the exposure to *Str. californicus*. Exposure to fungal species caused cell death similarly in human cell lines as in mouse macrophages, except for *S. chartarum* which was not toxic to human epithelial cells (Table 8).
Figure 2. Time-course of the relative concentrations of cytokines IL-6, TNF-α and IL-1β in mouse RAW264.7 macrophages, human 28SC macrophages and human A549 lung epithelial cells after exposure to dose of $10^6$ spores/ml of *Streptomyces californicus*. Data is presented as cytokine concentration divided by the average of the control level.
5.2 Differences between microbes (Studies I-III and V)

The concentrations of IL-6 and cytotoxicity after the exposure to three doses of each microbe in three cell lines (combined data from studies I-III) are presented in Table 8.

5.2.1 Production of inflammatory mediators

The inflammatory potency of different microbial species (III) and even the strains of the same species (I) varied significantly. On the basis of equivalent numbers of bacteria and spores of fungi, the overall potency to stimulate the production of proinflammatory mediators decreased in the order *Pseudomonas fluorescens* > *Streptomyces californicus* > *Mycobacterium terrae* > *Bacillus cereus* > *Mycobacterium avium* > *Stachybotrys chartarum* > *Aspergillus versicolor* > *Penicillium spinulosum*. Bacterial species were 100-1000 times more potent than fungal species in terms of their ability to evoke the production of inflammatory mediators (Table 8).

5.2.2 Cytotoxicity

In general, the cytotoxicity induced by the studied microbes followed their potency to produce inflammatory mediators. There were some exceptions, e.g. some fungal species were cytotoxic to the exposed cells even though they did not induce significant production of inflammatory mediators, and mycobacteria were less toxic than the other bacteria. The overall toxicity of the studied microbes decreased in the order *Pseudomonas fluorescens* > *Stachybotrys chartarum* > *Streptomyces californicus* > *Aspergillus versicolor* > *Bacillus cereus* > *Penicillium spinulosum* > *Mycobacterium avium* > *Mycobacterium terrae* (Table 8).
Table 8. Summary of the cytotoxicity and production of IL-6 after exposure to three doses of different microbes. A statistically significant (p<0.05) increase compared to control is indicated in bold font. Shaded cells show the doses exceeding the approximated overloading volumetric dose.

<table>
<thead>
<tr>
<th>Exposure agent</th>
<th>Dose (µg)</th>
<th>IL-6 (pg/ml)</th>
<th>Cytotoxicity (% of dead cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n = 6)</td>
<td></td>
<td>RAW 264.7</td>
<td>28SC</td>
</tr>
<tr>
<td>Buffer control</td>
<td>50 µl</td>
<td>3 ± 1</td>
<td>BD</td>
</tr>
<tr>
<td>LPS</td>
<td>10 µg</td>
<td>2350 ± 80</td>
<td>624 ± 20</td>
</tr>
<tr>
<td>Fungi (n = 3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillus versicolor</td>
<td>10³</td>
<td>BD</td>
<td>2 ± 1</td>
</tr>
<tr>
<td></td>
<td>10⁴</td>
<td>BD</td>
<td>2 ± 1</td>
</tr>
<tr>
<td></td>
<td>10⁵</td>
<td>BD</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Penicillium spinulosum</td>
<td>10³</td>
<td>BD</td>
<td>2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>10⁴</td>
<td>BD</td>
<td>1 ± 1</td>
</tr>
<tr>
<td></td>
<td>10⁵</td>
<td>2 ± 1</td>
<td>2 ± 0.3</td>
</tr>
<tr>
<td>Stachybotrys chartarum</td>
<td>10³</td>
<td>BD</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>10⁴</td>
<td>8 ± 4</td>
<td>3 ± 1</td>
</tr>
<tr>
<td></td>
<td>10⁵</td>
<td>NM</td>
<td>NM</td>
</tr>
<tr>
<td>Bacteria (n = 3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>10³</td>
<td>6 ± 2</td>
<td>2 ± 1</td>
</tr>
<tr>
<td></td>
<td>10⁴</td>
<td>110 ± 50</td>
<td>2 ± 1</td>
</tr>
<tr>
<td></td>
<td>10⁵</td>
<td>1400 ± 340</td>
<td>2 ± 0.3</td>
</tr>
<tr>
<td>Streptomyces californicus</td>
<td>10³</td>
<td>30 ± 8</td>
<td>4 ± 1</td>
</tr>
<tr>
<td></td>
<td>10⁴</td>
<td>2100 ± 40</td>
<td>14 ± 4</td>
</tr>
<tr>
<td></td>
<td>10⁵</td>
<td>2150 ± 40</td>
<td>170 ± 70</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>10³</td>
<td>2270 ± 50</td>
<td>30 ± 20</td>
</tr>
<tr>
<td></td>
<td>10⁴</td>
<td>2370 ± 20</td>
<td>340 ± 180</td>
</tr>
<tr>
<td></td>
<td>10⁵</td>
<td>2370 ± 30</td>
<td>480 ± 180</td>
</tr>
<tr>
<td>Mycobacteria (n = 3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycobacterium avium BA12</td>
<td>10³</td>
<td>3 ± 1</td>
<td>BD</td>
</tr>
<tr>
<td></td>
<td>10⁴</td>
<td>22 ± 6</td>
<td>BD</td>
</tr>
<tr>
<td></td>
<td>10⁵</td>
<td>420</td>
<td>2 ± 0.3</td>
</tr>
<tr>
<td>Mycobacterium avium BA31</td>
<td>10³</td>
<td>6 ± 1</td>
<td>BD</td>
</tr>
<tr>
<td></td>
<td>10⁴</td>
<td>300 ± 170</td>
<td>BD</td>
</tr>
<tr>
<td></td>
<td>10⁵</td>
<td>5150 ± 240</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Mycobacterium terrae BA20</td>
<td>10³</td>
<td>10 ± 3</td>
<td>BD</td>
</tr>
<tr>
<td></td>
<td>10⁴</td>
<td>250 ± 150</td>
<td>BD</td>
</tr>
<tr>
<td></td>
<td>10⁵</td>
<td>3460 ± 290</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Mycobacterium terrae BA 26</td>
<td>10³</td>
<td>80 ± 30</td>
<td>BD</td>
</tr>
<tr>
<td></td>
<td>10⁴</td>
<td>1600 ± 1100</td>
<td>BD</td>
</tr>
<tr>
<td></td>
<td>10⁵</td>
<td>5360 ± 460</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

LPS: lipopolysaccharide; BD: below detection limit 2 pg/ml; NM: not measured; n: number of independent experiments included in analyses; RAW264.7: mouse macrophages; 28SC: human macrophages; A549: human lung epithelial cells

* with mycobacteria, doses used with RAW264.7 cell line ranged between 5x10³-5x10⁷ instead of 10³-10⁷.
5.3 Differences between fungal metabolites (Studies IV, V)

The concentrations of cytokine TNF-α, and cytotoxicity caused by the exposure to one dose of each metabolite in studies I-II and V are presented in Table 9.

5.3.1 Production of inflammatory mediators

Exposure to fungal metabolites alone did not induce markedly increased production of NO, or IL-6. Only trichodermin, 7-α-hydroxytrichodermin and to some extent, ochratoxin A increased the production of the pro-inflammatory cytokine TNF-α (Table 9). The inflammatory potential of non-toxic S. chartarum strains was not associated with the production of atranones in study IV.

5.3.2 Cytotoxicity

Most cytotoxic metabolites were macrocyclic trichotheccenes produced by S. chartarum, namely satratoxins G, F and verrucarin J, followed by the less toxic metabolites trichodermin, 7-α-hydroxytrichodermin, staplabin and SMTP-7 (Table 9). The toxicity of S. chartarum - strains was associated with the production of macrocyclic trichotheccenes in study IV.

Table 9. Summary of the cytotoxicity and production of TNF-α in mouse RAW264.7 macrophages after exposure to 100 ng/ml of different fungal metabolites. A statistically significant (p<0.05) increase compared to control is indicated in bold font.

<table>
<thead>
<tr>
<th>Control</th>
<th>Exposure agent</th>
<th>TNF-α (pg/ml)</th>
<th>Cytotoxicity (% of dead cells)</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolites of Stachybotrys and relative species</td>
<td>HBSS/MeOH</td>
<td>130 ± 12</td>
<td>0</td>
<td>IV,V</td>
</tr>
<tr>
<td></td>
<td>Verrucarin J*</td>
<td>60 ± 20</td>
<td>98 ± 0.1</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>Iso-Satratoxin F*</td>
<td>50 ± 20</td>
<td>98 ± 0.4</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>Satratoxin G</td>
<td>80 ± 25</td>
<td>97 ± 1</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>Atranone B</td>
<td>110 ± 30</td>
<td>14 ± 8</td>
<td>IV,V</td>
</tr>
<tr>
<td></td>
<td>Atranone D*</td>
<td>110 ± 20</td>
<td>14 ± 7</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>Atranone E</td>
<td>110 ± 30</td>
<td>8 ± 5</td>
<td>V</td>
</tr>
<tr>
<td>Trichodermin</td>
<td>5500 ± 3200</td>
<td>50 ± 6</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>7-α-hydroxytrichodermin</td>
<td>140 ± 30</td>
<td>13 ± 6</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>Staplabin</td>
<td>72 ± 5</td>
<td>24 ± 5</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>SMTP-7</td>
<td>100 ± 5</td>
<td>25 ± 3</td>
<td>V</td>
<td></td>
</tr>
</tbody>
</table>

Mycotoxins produced by other fungi

| Sterigmatocystin | 120 ± 40       | 10 ± 10          | V                             |       |
| Citrinin         | 110 ± 20       | 13 ± 4           | V                             |       |
| Ochratoxin A     | 240 ± 60       | -1 ± 12          | V                             |       |

* doses of Verrucarin J and Iso-satratoxin F 70 ng/ml, atranone D 500 ng/ml instead of 100 ng/ml.
5.4 Synergistic interaction (Study V)

The synergistic effect of simultaneous exposure to *Streptomyces californicus* and *Stachybotrys chartarum* is illustrated in Figure 3, and *Str. californicus* together with trichodermin in Figure 4. Figure 5 depicts the cytotoxicity of the exposure to trichodermin together with *Str. californicus*.

5.4.1 Production of inflammatory mediators

Exposure to *Str. californicus* together with *S. chartarum* caused a synergistic response in the mouse macrophages. The synergistic response was seen in IL-6 production, where the combination of the two exposures increased the response by 30 fold. TNF-α production was increased without evidence of any clear synergistic interaction (Figure 3). A similar, but even more pronounced potentiation, was caused by simultaneous exposure to *Str. californicus* and trichodermin, and its hydroxylation product 7-α-hydroxytrichodermol. In contrast to the effects of the co-exposure to microbes, synergism was observed here also in TNF-α production, which increased to 2.5 fold during the simultaneous exposure accompanied by a 10 fold increase in IL-6 production (Figure 4).

5.4.2 Cytotoxicity

Co-exposure had a synergistic effect also on the cytotoxicity, although it was not as evident as the increase in inflammatory potency. Weak synergistic effect was detected with co-exposure to combinations of microbes at the low doses, and out of the studied fungal metabolites, trichodermin, 7-α-hydroxytrichodermol and satratoxin G increased slightly, but nonsignificantly the toxicity of the exposure to *Str. californicus* (Figure 5).

5.4.3 Nuclear binding activity of NF-κB

Exposure to *Str. californicus* increased the binding of NF-κB proteins to the NF-κB consensus sequence as well as to the natural NF-κB site of IL-6 promoter. Addition of trichodermin to the exposure did not increase the extent of the DNA binding (V).
**Figure 3.** Dose-response of the cytokines TNF-α (A) and IL-6 (B) in mouse RAW264.7 macrophages after exposure to doses of 10^5 spores/ml of *Streptomyces californicus* alone and together with three doses of *Stachybotrys chartarum*. Each column represents mean + SEM of three independent experiments. C: Buffer control; Stre: *Str. californicus.*
Figure 4. Dose-response of the cytokines TNF-α (A) and IL-6 (B) in mouse RAW264.7 macrophages after exposure to dose of $10^5$ spores/ml of *Streptomyces californicus* alone and together with six doses of trichodermin. Each column represents mean + SEM of five independent experiments. C: Buffer control; Stre: *Str. californicus*.
Figure 5. The cytotoxicity in mouse RAW264.7 macrophages caused by exposure to dose of $10^5$ spores/ml of *Streptomyces californicus* alone and together with six doses of trichodermin. Each column represents mean ± SEM of five independent experiments. C: Buffer control; Stre: *Str. californicus*. 
6. DISCUSSION

Previous research has indicated that microbial components may have both immunopotentiating and immunosuppressive effects, along with the cytotoxic capacity of certain metabolites produced by fungi and bacteria. On the other hand, the symptoms of the individuals exposed to a moisture damaged environment suggest that excessive inflammatory reaction or impairment of immune defense participate in the progression of the disorders. However, the prevalence of the health effects is not directly dependent on the observed concentration of indoor air microbes (Verhoeff and Burge 1997), thus the active components of microbial exposure may be associated with only certain microbes or environmental conditions. Recognition of these etiological factors would enable priority listing of the repairs and possibly help the diagnosis of the patients suffering from moldy house exposure. The diversity of microbial species and other exposure agents in a moisture damaged environment complicates the comparison between different elements of exposure, but there is a good amount of descriptive data gathered from mold problem buildings, which provides a sound basis for toxicological studies.

6.1 Characteristics of the studied cell lines

The epithelial lining of the lung is the cell type which is the first to encounter the pollutants in the inhaled air. In this study, the human lung epithelial cell line responded weakly to the microbial exposure, although the inflammatory and cytotoxic response of the cells differed from the control. The spores of \textit{Str. californicus}, LPS, \textit{P. fluorescens}, and mycobacterial cells triggered an IL-6 response in these cells, whereas TNF-\(\alpha\) was not detected and the production of nitrite was generally low. The A549 lung epithelial cell line is a well known model for research of inhalation exposure. It has been used in studies examining the inflammatory potential of dust from waste handling facilities and schools (Meyer et al. 1999, Allerman and Poulsen 2000), biological particles (Palmberg et al. 1998), swine dust (Wang et al. 1999), actinomycete \textit{Streptomyces anulatus} (Jussila et al. 1999), house dust mite allergens (Asokananthan et al. 2002) and cytotoxicity of microbial volatile organic compounds (Kreja and Seidel 2002). Altogether, these studies show that these cells are able to produce some inflammatory agents e.g. the chemokine IL-8 and cytokines IL-6 and IL-1, but their ability to produce TNF-\(\alpha\) is not established (Spriggs et al. 1988), although it has been measured at the transcriptional level (Yang et al. 2003).
The human macrophages used in this study responded most clearly to the spores of Str. californicus, P. fluorescens and LPS similarly as the human epithelial cells and mouse macrophages. In general, only low levels of the produced inflammatory mediators were detected, indicating that human macrophages in this in vitro model need greater refining in order to function more efficiently. In general, macrophages that are derived from blood monocytes are less efficient phagocytes than the already differentiated alveolar macrophages. Macrophages have an essential role in the immune defense, phagocytosing the invading particles and activating other immunocytes. Human monocytic cell lines such as U937 and THP-1 have been used in the assessment of the inflammatory potential of indoor air dust (Allermann and Poulsen 2002), but there are no previous studies published on 28SC macrophages.

The enzyme iNOS was not detectable in the human cell lines, but correspondingly, only low levels of produced nitrite were detected. The ability of the human macrophages to produce NO is still controversial, since the production is elevated in infected patients but it has not been possible to induce NO production in vitro (Fang 1997). It has been proposed that the in vitro models of human macrophage function are incomplete, missing some fundamental component of activation (Bonecini-Almeida et al. 1998).

Mouse macrophages were the most sensitive of the used cell lines, mounting at least 100 times greater cytokine response than the human cell lines to the same doses of microbes. In addition, the expression of iNOS was detected when markedly increased nitrite concentrations were measured in the culture medium. The toxic effects of the microbial exposure were also more prominent in mouse cells compared to human cell lines. On the other hand, all the cell lines responded to some extent to the same stimuli, despite the differences in the levels and profiles of the produced cytokines. The mouse RAW264.7 macrophage cell line is a widely used model for evaluating murine responses to a variety of stimuli; the effects of microbes, toxins, LPS and other mitogens have all been examined using these cells (Hirvonen et al. 1997, Rotter and Oh 1996, Ji et al. 1998, Wong et al. 1998). Thus, mouse macrophages seem to be a sensitive and useful cell line model for studies of microbial exposure.

Some of the microbes studied here (Str. californicus, M. terrae, A. fluorescens, P. spinulosum) have been recently studied also in a mouse model in vivo (Jussila 2003), which confirmed the inflammatory potential observed in the current in vitro studies. The similarities
in the responses of the human and mouse macrophage cell lines suggest that the responses seen in the mouse model could be relevant in humans as well. This assumption is further supported by the clinical studies on people exposed in moisture and mold damaged schools and other buildings. The studies described above show that the increased production of the same inflammatory mediators can be detected also in the nasal lavage fluid of the exposed individuals during the exposure period in a moisture damaged work environment, but not during vacations (Roponen 2002).

6.2 Validity of the selected doses

When studying multiple endpoints of exposure, it is necessary to use several dose levels and a range of exposure times in order to determine the dose response curve and a relevant time point for analysis of each marker. However, comparison of the different species of microbes is problematical, since the dose could be defined either as a mass, volume, surface area, or number of microbes. Depending on the size of the microbe, the amount of microbial mass may differ considerably even though the number of microbes is equal. For this reason, it should be noted that the doses used in this study are based on equivalent numbers of spores/cells and thus the comparison between bacteria and fungi is confounded by the typically larger particle size of the fungal species.

Furthermore, excessive volumetric dose of particles may cause phagocytic cells to overload, in other words, a high amount of particles can cause non-specific activation of the macrophages, regardless of the exposure agent. Overloading effect has been observed in rat lungs at a dose of 60 µm³/alveolar macrophage (Morrow 1988), thus the approximated volumetric doses used in this study exceed the limit of overloading in the case of the highest tested doses of fungal spores. In the current study, the bacterial species were in general more potent than fungi despite the smaller volumetric dose, thus the comparison based on equal numbers does not distort the results.

The doses of the mycotoxins used in these experiments relate to the concentrations of microbes, considering that the approximated levels of macrocyclic trichotheccenes is around 140 fg/spore (Nikulin et al. 1996). Hence the dose of 100 ng/ml can be estimated to correspond a dose of $10^6$ spores/ml, which was the highest dose used in the interaction studies.
Both epithelial cells and macrophages are able to take up the microbial spores, although the phagocytes are more efficient both at internalizing and destroying the biological particles. The rate of uptake may explain the relatively low sensitivity of epithelial cells (WasylNka and Moore 2002). The current results show that the characteristics of the phagocytosed particle determines the response of the cell, thus the particle-effect as such does not explain the observed inflammatory responses. Particle-induced TNF-α production has been studied earlier with different exposure agents, and the study concluded that non-reactive exposure agents such as latex beads or titanium oxide induce only minor responses in the phagocytes, whereas biologically active components cause intense production of TNF-α (Driscoll et al. 1997).

6.3 Differences in the inflammatory and cytotoxic potency of microbial exposures

The exposure to the microbes provoked inflammatory changes in the exposed human and mouse cells, but there were considerable differences in the potency of the microbial species and strains. Overall, the bacteria were more potent than the fungi to induce the production of inflammatory mediators and associated cytotoxicity, whereas some of the tested fungal strains caused cytotoxicity without any major production of inflammatory mediators.

In addition to the difference between the microbial species, the inflammatory potential differed also between two strains of the same species when the mycobacterial isolates were studied (I). This could be due to different chemical composition of the active component in microbial cell wall, which has adapted to the specific environmental conditions. Previously, the differences in the immunogenicity of mycobacterial strains has been studied in the context of the evaluation of virulence. These studies have shown that the mannosylation of the mycobacterial cell wall component lipoarabinomannan can influence the inflammatory potential (Venisse et al 1995), thus the differences observed in this study may originate from similar changes in the chemical composition. This notion emphasizes the fact that the characteristics of the microbial exposure should be studied thoroughly before making any conclusions of the outcomes. The variability between strains also supports the hypothesis that the growth environment of the microbe determines its characteristics, including its inflammatory potential (Hirvonen et al. 2001, Murtoniemi et al. 2001, Roponen et al. 2001).
The most potent of the studied bacteria in the mouse macrophages was the gram negative bacterium *Pseudomonas fluorescens* (II, III). The inflammatory potency of this species is apparently derived from the cell wall component LPS, which is readily recognized by the phagocytes. Interestingly, gram negative bacteria are not the only bacterial cell type causing an inflammatory response, since the gram positive bacterium *Streptomyces californicus* proved to be almost as potent in these studies, and even more potent in human epithelial cell line. Gram positive bacteria lack the LPS in their cell wall structure, thus the active component in this microbe is not known. However, strong inflammatory potency is not typical for all gram positive bacteria, because *Bacillus cereus* induced only minor responses compared to *Streptomyces californicus* and mycobacterial strains. All the studied bacterial species are approximately of the same size, thus the doses are comparable and the differences in the responses must be associated with the properties of the microbes, not the particle effect.

The current findings are of special interest, since the elevated levels of viable airborne bacteria in buildings with water damage have been reported to be associated with an increase in the level of reported symptoms (Teeuw et al. 1994). Bacterial lipopolysaccharide has been proposed to be the etiological factor in building related illnesses, although increased levels of gram positive bacteria have also been reported in problem buildings (Li et al. 1997a, Sudakin 1998).

The fungal strains examined in this study caused rather weak inflammatory responses compared to bacteria (III). However, it is possible that the characteristics of the fungi, especially the produced metabolite profile is highly dependent on the growth environment of the microbe (Nielsen et al. 1998, Murtoniemi et al. 2001). The strains used in this study were isolated from an indoor environment, but subsequent cultivation on standard media possibly alters the metabolic state of the microbe. The observed link between the cytotoxicity and metabolic profile of the strains of *Stachybotrys* further confirmed the theory that the metabolites produced by the fungi define its toxicity, although the inflammatory potency of the non-toxic strains could not be explained by the effects of the studied metabolites (IV).
6.4 Microbial interactions

Due to the major inflammatory and cytotoxic potential of the spores of the actinomycete *Streptomyces californicus*, interactions of this microbe together with the other microbes were studied. The tested combinations of moldy house microbes in this study revealed that the low dose of spores of *Streptomyces californicus* together with the spores of the fungus *Stachybotrys chartarum* induced a synergistic, dose dependent increase in inflammatory responses indicated as production of IL-6, whereas other tested microbes caused merely additive or non-significant responses (V). This finding is of special interest, since these particular microbes are often found together in moldy houses, especially from paper materials. Other microbes frequently found together in moldy houses are actinomycetes and *Acremonium* (Hyvärinen et al. 2002), but the effect of the co-exposure to these species is not known. Interestingly, a similar synergistic effect was found when the metabolites typically produced by *Stachybotrys chartarum* were tested together with the same low dose of spores of *Str. californicus*. Seemingly innocuous doses of trichodermin, a known protein synthesis inhibitor produced by *Stachybotrys* spp. caused a marked increase in both TNF-α and IL-6 production, and the effect could be seen also in cytotoxicity caused by the co-exposure. Nevertheless, trichodermin is not the only compound capable of this phenomenon, since also the closely related 7-α-hydroxytrichodermin caused a similar synergistic increase. In line with the present findings, there are previous reports showing that there are other mycotoxins which have a similar effect together with gram negative bacterial LPS (Ji et al. 1998, Wong et al. 1998).

To further study the mechanisms behind the detected synergistic increase in inflammatory responses, the nuclear binding activity of the transcription factor NF-κB was studied. The increased translocation of the NF-κB to the nucleus after exposure to the spores of *Str. californicus* indicates that this moldy house microbe is recognized by the macrophages. Consequently the signal transduction is activated, leading to the expression of the genes involved in inflammation. The synergistic increase in IL-6 production in co-exposure to actinomycete and trichodermin could not be explained by any increased amount of the NF-κB in the nucleus. However, the increase in the level of this cytokine may be affected by many other factors such as binding of other transcription factors to IL-6 promoter, increase in the stability of the produced mRNA, or inhibition of negative regulatory elements of the signal
transduction. These mechanisms have been shown to be associated with the superinduction of cytokines caused by trichothecene DON in PMA and ionomycin-stimulated murine EL-4 lymphoma cell line (Ouyang et al. 1996, Li et al. 1997b, Yang and Pestka 2002), and LPS stimulated mouse RAW264.7 macrophages (Wong et al. 2001, 2002).

The receptors for these inflammatory signals need to be clarified in further studies, since the recognition of the involved receptors and their activation would shed light on the mechanism by which the moldy house microbes interact with the host cells. Altered or defective expression of the receptors would also explain the variability in the sensitivity of the exposed people, since mutations in PRRs have been suggested to account for the differences in responsiveness to microbial challenge as modeled with LPS (Schwartz 2001).

The exposure occurring in moldy houses is a combination of several components. Different exposure agents may interact either during the growth of the microbes (e.g., biocides) or during the exposure. The secondary metabolites produced by fungi to defend their habitat from other microbes may inadvertently cause toxic effects also outside the actual targets. The results of the present study suggest that the simultaneous exposure to multiple agents is a possible explanation for the amplified health effects in a moisture damaged environment. Thus even at relatively low concentrations of each individual exposure agent, the synergism between them may multiply the overall effects.

6.5 The value of in vitro-studies in environmental toxicology

In vitro studies utilize cell cultures when studying biological phenomena. Continuous cell cultures are a cost effective, reproducible and ethical method of studying the response of living cells in a controlled exposure environment. The disadvantages of the method include lack of complexity of a living organism and its feedback systems, which may be a source of bias, and this limits the applicability of the results in risk assessment or in evaluation of their clinical implications. Studies involving a large number of samples (screening) or requiring a high degree of specificity (mechanistic studies) are exceptionally well suited for in vitro work. In order to undertake a valid risk assessment, the findings of in vitro studies have to be confirmed both in vivo and with epidemiological data, but a well conducted cell culture study with carefully selected cell types is a good foundation on which to base further research.
7. CONCLUSIONS

1. Mycobacteria isolated from a moldy building were capable of inducing the production of inflammatory mediators in vitro. Even strains of the same species differed in their ability to evoke these responses.

2. Mouse cells were clearly more sensitive to the microbial exposure than the human cell lines. However, the microbial stimuli induced a response both in mouse and human cells, with IL-6 being the most consistent marker in the different cell lines. Production of inflammatory cytokines could be observed already after a few hours of exposure, and each measured marker possessed its own distinct time scale and intensity of the production.

3. Moldy house bacteria were more potent than fungi at inducing the production of inflammatory markers, although many fungal species may have toxic effects other than those caused by the inflammatory reaction.

4. The toxicity of a fungal strain was associated with the produced metabolite profile, although its biological activity may not be solely dependent on the toxin production.

5. Simultaneous exposure to certain microbes can evoke an enhanced inflammatory response in the exposed cells. A synergistic increase could be seen also when using combinations of biologically active metabolites.
8. REFERENCES


61


62


Wong SS, Zhou HR, Marin-Martinez ML, Brooks K and Pestka JJ. 1998. Modulation of IL-1, IL-6 and TNF secretion and mRNA expression by the trichothecene vomitoxin in the RAW264.7 murine macrophage cell line. Food and Chemical Toxicology 36:409-419.


Inflammatory responses in RAW264.7 macrophages caused by mycobacteria isolated from moldy houses

Kati Huttunen a,*, Marjo Ruotsalainen a, Eila Iivanainen b, Pirjo Torkko b, Marja-Leena Katila c, Maija-Riitta Hirvonen a

a Laboratory of Toxicology, Division of Environmental Health, National Public Health Institute, P.O. Box 95, FIN-70701 Kuopio, Finland
b Laboratory of Environmental Microbiology, National Public Health Institute, P.O. Box 95, FIN-70701 Kuopio, Finland
c Department of Clinical Microbiology, Kuopio University Hospital, P.O. Box 1777, FIN-70211 Kuopio, Finland

Received 23 February 2000; received in revised form 9 May 2000; accepted 22 May 2000

Abstract

Mycobacterial strains (nonpathogenic Mycobacterium terrae, potentially pathogenic Mycobacterium avium-complex and Mycobacterium scrofulaceum), isolated from a moldy building, were studied with respect to their ability to stimulate macrophages (RAW264.7) to produce inflammatory mediators, and to cause cytotoxicity. Reactive oxygen species (ROS) were measured by chemiluminescence, cytokines (TNF-α, IL-6, IL-1, IL-10) immunochemically, nitric oxide (NO) by Griess-method, expression of inducible NO-synthase (iNOS) with Western Blot analysis and cytotoxicity with MTT-test. All the strains induced dose- and time-dependent production of NO, IL-6 and TNF-α in macrophages, whereas IL-1 or IL-10 production was not detected. The production of ROS and cytotoxicity was increased with the highest doses. Interestingly, different strains had significant differences in their ability to induce these responses, M. terrae being the most potent and M. avium-complex the weakest one. These results indicate that both non- and potentially pathogenic strains of mycobacteria present in moldy buildings are capable of activating inflammatory mechanisms in macrophages. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Environmental mycobacteria; Indoor air; NO; Cytokines; Macrophages

1. Introduction

An association between moldy microbial growth in buildings and respiratory symptoms in the inhabitants has been shown in several epidemiological studies. A variety of symptoms are related to inflammatory reactions e.g. irritation, respiratory infections, wheeze, bronchitis and even asthma (Platt et al., 1989; Waegemaekers et al., 1989; Brunekeef, 1992; Spengler et al., 1994). This suggests that inflammatory responses toward specific structural components or metabolic products of microbes present in the moldy buildings may contribute to the observed respiratory symptoms. However, the identification of the most potent microbes and specific cellular mechanisms responsible for the adverse health effects are not yet known.

Recent studies have shown that environmental mycobacteria can be isolated from indoor air and building materials of moldy houses (Andersson et al., 1997). Environmental mycobacteria include both potentially pathogenic and saprophytic species, which share the unique feature of all mycobacteria; a thick, hydrophobic and lipid-rich cell wall, also containing serologically active polysaccharides (Falkinham, 1996; Chatterjee and Khoo, 1998; Goodfellow and Magee, 1998). The cell wall structure enables mycobacteria to survive and even proliferate in extreme environments, such as in areas contaminated with xenobiotics or inside of host macrophages. The pathogenicity of mycobacteria such as the notorious Mycobacterium tuberculosis and Mycobacterium leprae is based on the immunological responses they elicit in the host, beginning with an ineffective elimination of the bacteria, and ending with the tissue-damaging hypersensitivity reactions (Clark-Curtiss, 1998). Some environmental mycobacteria have also been recognized as effective immunostimulators in
murine models (Bradbury and Moreno, 1993; Denis and Ghadriam, 1994; Falcone et al., 1994; Sarmento and Appelberg, 1995). Mycobacteria present in moldy buildings are of special interest, because they are inhalable particles and have potential to stimulate a cascade of cytokines, leading to recruitment and activation of inflammatory cells.

Proinflammatory cytokines (e.g. IL-6, IL-1 and TNF-α) are known to initiate the expression of inducible nitric oxide synthase (iNOS) (Moncada and Higgs, 1993), whereas anti-inflammatory cytokines (e.g. IL-10) tend to suppress the inflammatory responses (Marshall et al., 1997). In a wide variety of cells in the respiratory tract, iNOS catalyzes the production of nitric oxide (NO) using L-arginine as a source. The role of nitric oxide as an immune defense molecule and important mediator in airway diseases together with reactive oxygen species (ROS) has been confirmed by an increasing amount of information during this decade (Fridovich, 1995; Singh and Evans, 1997).

In our previous studies we have shown that other gram positive bacteria Streptomyces sp., particularly present in moldy houses, induce the production of inflammatory mediators (cytokines, NO and ROS) in murine macrophages. In contrast, bacterial strains belonging to normal microbial flora of indoor air do not cause similar responses (Hirvonen et al., 1997a,b,c). Thus, the role of environmental mycobacteria as immunostimulators clearly needs further clarification, since exposure to these bacteria in moldy houses could be one of the etiological factors leading to the respiratory symptoms observed in the inhabitants.

In the present study we investigated whether five strains of environmental mycobacteria, isolated from indoor air in moldy buildings during demolition work, are able to stimulate mouse macrophages (RAW264.7) to produce inflammatory mediators (i.e. TNF-α, IL-6, IL-1, IL-10, iNOS, NO, ROS), and to cause cytotoxicity in these cells.

2. Materials and methods

2.1. Mycobacteria

Five mycobacterial strains were isolated from a moldy building during the demolition work (Rautiala et al., 1995). The strains were identified by using gas liquid chromatography (GLC) of fatty acids and alcohols of isolated strains. In addition, tests for growth and biochemical characteristics and commercial DNA probe analyses (AccuProbe; GenProbe Inc., San Diego, CA) were performed as described previously (Torkko et al., 1998). Strains belong to three different species; potentially pathogenic Mycobacterium avium-complex (BA12 and BA31), Mycobacterium xenopi [BA24] and nonpathogenic Mycobacterium terrae [BA20 and BA26]. For the present study, the mycobacterial strains were cultured on Mycobacteria 711-agar supplemented with OADC-enrichment (Difco Laboratories, Detroit, MI) at 30°C. After 5 weeks, the bacterial cells were washed, suspended in Hank's Balanced Salt Solution (HBSS) (Gibco BRL, Paisley, UK) and sonicated for 15 min. Concentrations were counted at 1250 × magnification using an epifluorescence microscope (Olympus TSH2-RFL, Olympus Optical Co., Tokyo, Japan) and direct acridine orange staining modified for mycobacteria (Hobbe et al., 1977; Katila and Mäntyläri, 1982).

2.2. Cell culture

Mouse macrophage cell line RAW264.7 was obtained from American Type Culture Collection, Rockville, MD. The cells were cultured at 37°C in 5% CO₂ in RPMI 1640 medium supplemented with 10% of heat inactivated fetal bovine serum (FBS), 1% of l-glutamine and 1% of Penicillin-Streptomycin (all from Gibco BRL, Paisley, UK). Macrophages were dispensed to six-well plates, 5 × 10⁵ cells/ml, 2 ml/well. They were allowed to adhere for 24 h, fresh complete RPMI medium was added and cells were exposed to six doses (5 × 10⁴, 5 × 10⁵, 5 × 10⁶, 5 × 10⁷, 5 × 10⁸, and 5 × 10⁹) mycobacterial cells/ml of the five mycobacterial strains for 24 h. The time dependency of the induced responses was studied at six timepoints (2, 4, 8, 16, 24 and 48 h) after the exposure to non cytotoxic dose of 5 × 10⁴ mycobacterial cells/ml. After the exposure, the cells were resuspended in the culture media and the cell suspension was centrifuged (5 mm, 8000 rpm) in order to separate the cells from the culture media. The supernatants were stored at −80°C for the analyses of cytokines and the cells were frozen immediately in dry ice and stored at −80°C for Western blot analysis.

2.3. Nitrite analysis

Nitric oxide (NO) was measured spectrophotometrically as the stable metabolite, nitrite (NO₂⁻) according to the Griess method (Green et al., 1982). Briefly, Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% phosphoric acid) was mixed 1:1 with samples of the cell culture medium. Nitrite forms a colored chromophore with the reagent, with an absorbance maximum at wave length of 543 nm, which was measured with a ELISA microplate reader (iEMS Reader MF, Labsystems, Turku, Finland). The production of nitrite was quantified by comparing the results with absorbencies of standard solutions of sodium nitrite (NaNO₂). Samples of three independent experiments were analyzed in duplicate.
2.4. Western Blot analysis

Expression of the inducible NO-synthase (iNOS), the enzyme that catalyses the NO production, was analyzed with Western Blot technique. The frozen cells were lysed in lysis buffer (1 mM ethylenediaminetetraacetic acid (EDTA); 50 mM Tris(hydroxymethyl)-amino sulphoxylate (Tris–HCl); 0.1 mM phenylmethylsulphonyl fluoride (PMSF) dissolved in ethanol; 10 μg/ml leupeptin) with the help of an ultrasound sonicator. Cell fragments were centrifuged (9000 rpm) for 10 min. Sample buffer (3 mM Bromophenol Blue; 0.28 M sodium dodecyl sulphate (SDS); 0.25 M Tris–HCl; 0.2 M β-mercaptoethanol; 2.7 M glycerol) was added 1:4 to the supernatant and heated at 95°C for 8 min. Samples were subjected to electrophoresis through 7.5% Tris–glycine gel with 4% stacking gel (Bio-Rad Laboratories, Hercules, CA) in order to differentiate proteins by their size. From the gel, proteins were transferred electrophoretically to a nitrocellulose membrane.

Membranes were incubated overnight at 4°C in blocking buffer (100 mM Tris–HCl; 150 mM NaCl; 30 g/l albumin; pH 7.4), rinsed with washing buffer (10 mM Tris–HCl; 150 mM NaCl; 0.1% Tween; pH 7.4) and incubated in primary antibody solution (0.1% rabbit anti-iNOS (Santa Cruz Biotechnology, CA) in washing buffer) for 2 h at room temperature. After primary antibody, the membranes were washed (3 × 10 min) with washing buffer and incubated in secondary antibody solution (0.05% alkaline phosphatase conjugated Goat Anti-Rabbit IgG (Bio-Rad Laboratories, Hercules, CA) in washing buffer) for 45 min. The membranes were washed again, bathed in developing buffer (100 mM NaCl; 100 mM Tris; 50 mM MgCl₂ × 6H₂O) and developed in developing solution (330 mg/l nitro blue tetrazolium (NBT); 165 mg/l bromochlorindolyl phosphate disodium (BCIP) (both from Sigma Chemical Co., MO) in developing buffer). Colored bands at 130 kD were visually detected from the membrane.

2.5. Cytokine analysis

TNF-α, IL-6, and IL-10 were analyzed from the cell culture medium immunochemically with commercial ELISA kits (Pharmingen, San Diego, CA) as described earlier (Ruotsalainen et al., 1998), and IL-1β with a similar product, DuoSet™ (Genzyme, Cambridge, MA). Samples were processed according to the manufacturer’s protocol and analyzed with the ELISA microplate reader by comparing the absorbencies of the samples to the standard curve. Samples of three independent experiments were analyzed in duplicate.

2.6. Reactive oxygen species

The production of ROS was assayed by a luminometric method as described earlier (Ruotsalainen et al., 1998). Briefly, mycobacterial cells were opsonized with FBS at 37°C for 30 min, washed and suspended in HBSS. The macrophages were exposed to opsonized mycobacterial cells at increasing doses from 10⁴ to 10⁶ mycobacterial cells/ml in a luminol reaction mixture (70 μM 5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma Chemical Co., MO) and emission of light was measured immediately after exposure for 1 h at 37°C with Bio-Orbit 1251 luminometer (Bio-Orbit, Turku, Finland). Samples of four independent experiments were analyzed in duplicate.

2.7. Cell viability

Viability of the macrophages was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)-test to detect functioning mitochondria (Mosmann, 1983). Live mitochondria transform MTT (Sigma Chemical Co., MO) to formazan, which can be measured with a spectrophotometer. The proportion of viable cells in exposed samples was compared to control samples. In addition, viability of the macrophages in the control samples was estimated after staining the cells with Trypan Blue solution. Samples of at least three independent experiments were analyzed in duplicate.

2.8. Statistical analysis

Data were logarithmically transformed to match the normal distribution. The results were statistically analyzed with one-way variance analysis (SAS Institute Inc., 1989). P < 0.05 was considered statistically significant.

3. Results

3.1. Production of cytokines

All the five mycobacterial strains tested in this study induced dose-dependent increase in TNF-α (Fig. 1A) and IL-6 (Fig. 2A) production in the RAW264.7 cells. The most potent strain was Mycobacterium terrae (BA 26) inducing TNF-α and IL-6 production up to 2840 ± 980 pg/ml and 5360 ± 460 pg/ml, respectively. TNF-α production was slightly suppressed with the lowest doses, reaching statistical significance at the exposure to 5 × 10⁴ mycobacterial cells/ml of Mycobacterium scrofulaceum (BA24) and M. terrae (BA26). The Mycobacterium avium–complex (BA 12) caused only a minor increase in the IL-6 concentrations, and no increase in
the TNF-α production. In the time course study the production of TNF-α was seen as early as 2 h after the exposure to strains BA24, BA26 and BA31, and maximum production was obtained at 16 and 24 h. After 8 h, the TNF-α production caused by the most potent inducer M. terrae (BA 26) boosted to the upper detection limit (5460 ± 1540 pg/ml) until a decrease after 24 h (Fig. 1B). Increased IL-6 production in the exposed macrophages was detected after 8 h of exposure to all the strains and it further increased time-dependently until the end of the test (48 h) (Fig. 2B). M. terrae (BA 26) was the most potent inducer of IL-6 production, increasing IL-6 concentration up to 4700 ± 610 pg/ml (Fig. 2B). None of the tested strains of mycobacteria were able to cause significant production of IL-1β or anti-inflammatory cytokine IL-10.

3.2. Production of nitrite and expression of iNOS

The nitrite concentrations in the cell culture medium of the RAW264.7 macrophages was increased dose-dependently after the exposure to all the five mycobacterial strains when compared to controls (1.3 ± 0.1 μM) (Fig. 3A). In the dose–response experiment the most potent strain, M. terrae (BA26), increased NO2-production significantly already with the dose of 5 × 10⁵ mycobacterial cells/ml and the other tested strains with the dose of 5 × 10⁶ mycobacterial cells/ml. The highest doses of both strains of nonpathogenic M. terrae (BA 20 and BA 26) increased the NO2 concentration up to 42 ± 7 and 39 ± 1 μM, respectively. The highest concentrations of nitrite induced by both potentially pathogenic M. avium and M. scrofulaceum were 22 ± 1 μM.

Fig. 1. (A) Effects of graded doses of five mycobacterial strains on TNF-α production by RAW264.7 mouse macrophages after 24 h exposure. Internal graph shows a smaller concentration range for the first two doses. (B) Time course of TNF-α production after exposure to single doses (5 × 10⁸ bacteria cells/ml) of five mycobacterial strains. Inset shows a smaller concentration range for the first three time points. Each column represents mean ± SEM of three independent experiments done in duplicate. * indicates statistically significant difference from control, P < 0.05.
Fig. 2. (A) Effects of graded doses of five mycobacterial strains on IL-6 production by RAW264.7 mouse macrophages after 24 h exposure. Internal graph shows a smaller concentration range for the first four doses. (B) Time course of IL-6 production after exposure to single doses (5 × 10⁸ bacteria cells/ml) of five mycobacterial strains. Inset shows a smaller concentration range for the first three time points. Each column represents mean ± SEM of three independent experiments done in duplicate. * indicates statistically significant difference from control, P < 0.05.

In the time course study, the dose of 5 × 10⁸ mycobacterial cells/ml caused significant increase in the NO₂ production in macrophages compared to control cells after 8 h exposure to one strain of *M. terrae* (BA 26), and after 16 h exposure to the other tested strains except *M. avium*-complex (BA 12) (Fig. 3B). The expression of iNOS was detectable after 16 h exposure in all the samples in which the NO₂ concentration in the culture medium of the macrophages was markedly enhanced (Fig. 4).

3.3. Production of ROS

*M. terrae* (BA 26) was the most potent strain to induce ROS production in the RAW264.7 macrophages. The highest dose of mycobacteria (10⁸ mycobacterial cells/ml) increased the production of ROS from two to fourfold compared to control cells, except one strain of *M. avium*-complex (BA 12) which did not cause statistically significant increase in ROS production in these cells (Fig. 5).
3.4. Cell viability

Exposure to the highest dose (5 x 10^7 mycobacterial cells/ml) of strains M. scrofulaceum (BA 24), M. terrae (BA 26) and M. avium—complex (BA 31) decreased significantly the viability of RAW264.7 macrophages when compared to controls (Fig. 6). The most cytotoxic strain was M. terrae (BA 26), which decreased the viability of the cell population down to 50% with the highest dose in 24 h exposure.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>SAMPLE</th>
<th>DOSE (bacterial cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA 12</td>
<td>1</td>
<td>MARKER 130 KDa</td>
</tr>
<tr>
<td>BA 20</td>
<td>2</td>
<td>CONTROL</td>
</tr>
<tr>
<td>BA 24</td>
<td>3</td>
<td>5 x 10^4</td>
</tr>
<tr>
<td>BA 26</td>
<td>4</td>
<td>5 x 10^3</td>
</tr>
<tr>
<td>BA 31</td>
<td>5</td>
<td>5 x 10^4</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5 x 10^3</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>5 x 10^2</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>5 x 10^1</td>
</tr>
</tbody>
</table>

Fig. 4. Effects of graded doses of five mycobacterial strains on expression of iNOS in RAW264.7 macrophages after 24 h exposure.

4. Discussion

The present results demonstrate that environmental mycobacteria, isolated from the indoor air of a moldy building can be potent activators of inflammatory responses in mammalian cells. Among the strains analyzed this particularly applied to saprophytic species i.e. M. terrae (BA 20 and BA 26) and to M. scrofulaceum (BA 24) and M. avium (BA 31). These strains of mycobacteria stimulated RAW264.7 macrophages to produce dose- and time-dependently substantial amounts of NO and proinflammatory cytokines TNF-α and IL-6. Moreover, the production of ROS was induced by high mycobacterial doses. Interestingly, the
same concentrations of mycobacteria that initiated the series of inflammatory responses were not markedly cytotoxic, only the highest doses and extended exposure caused significant cell death.

In this study the first indicator of inflammatory activation of the RAW264.7 macrophages was the increase in TNF-α concentration in the cell culture medium. TNF-α was detected as early as after 2 h of exposure and the concentration increased until a decline after 24 h, probably as a result of its sensitivity to proteolytic enzymes. The TNF-α production in macrophages induced by mycobacteria originated from indoor air is of special interest since there is evidence of increased TNF-α expression in asthmatic animals (Staines et al., 1998). The important role of TNF-α in pathogenesis of mycobacterial infection has been shown in several earlier reports (Bradbury and Moreno, 1993; Kaufman, 1993; Falcone et al., 1994; Sarmento and Appelberg, 1995). Both human and mouse macrophages have been shown to produce TNF-α after the exposure to mycobacterial cell wall component lipoarabinomannan (LAM) (Moreno et al., 1989).

The IL-6 production increased few hours later than the TNF-α production, but once it was initiated, the concentration boosted rapidly to the upper limit of detection and further increased until the end of the follow-up. This observation is consistent with the assumption that IL-6 synthesis is secondarily activated by other proinflammatory cytokines e.g. TNF-α and IL-1 (Marshall et al., 1997). In the present study the mycobacterial stimulation increased IL-1β production only slightly and only with the highest doses used, suggesting that this experimental setting seems to favor the TNF-α pathway. IL-1β production may require a longer time to become initiated. This assumption is supported by a previous report showing that mouse peritoneal macrophages released IL-1 significantly at 4 and 7 days following infection with M. avium (Denis and Ghadririan, 1994). It has been shown that IL-6 activates B-lymphocytes to produce antibodies, and primes respiratory burst from neutrophils and monocytes (Chuzel and Lee, 1992). Moreover, IL-6 is known to be released in asthma although its specific role in respiratory diseases remains unclear (Barnes et al., 1998).

In the contrast to the production of proinflammatory cytokines, concentrations of anti-inflammatory cytokine IL-10 was only modestly increased compared to control cells, and this effect was not time or dose dependent. The role of IL-10 in mycobacterial challenge is somewhat controversial; IL-10 has been shown to decrease TNF-α production caused by M. smegmatis stimulation, although in some experiments it seems to amplify the effects of immunostimulation (Marshall et al., 1997).

Expression of iNOS in macrophages by all the tested strains of mycobacteria and subsequent production of substantial amount of NO in the RAW 264.7 cells is intriguing, because excessive production of NO is known to be involved in inflammatory airway diseases including asthma (Curran, 1996). Classical signs of inflammation, such as mucus secretion, eosinophilia and hyperemia in airways are associated with increased NO production (Barnes, 1995). In the present study, the mycobacteria induced also ROS-production in macrophages which may amplify the potential adverse effects, since NO reacts eagerly with reactive oxygen compounds forming extremely powerful oxidants such as peroxynitrite (ONOO-) (Tamir and Tannenbaum, 1996). There is also immunocytochemical evidence of peroxynitrite formation in asthmatic airways (Guidi et al., 1998). Although the cytotoxicity of the exposure was evident only with the highest doses, the initiation of the cascade leading to apoptosis with the lower doses is possible. This interpretation is supported by some previous evidence of the causal connection between mycobacterial infection and apoptosis (Rajasekhar, 1997).

Interestingly, different mycobacterial strains had significant differences in their ability to stimulate macrophages. A strain of nonpathogenic M. terrae (BA 26) was an exceptionally potent inducer of all measured inflammatory markers, and a strain of potentially pathogenic M. avium-complex (BA 12) was the weakest one. Even the different strains among the same mycobacterial species had different potency to trigger these responses in the exposed macrophages, indicating that growth environment of the microbe may be an important factor. In general, mycobacterial species that are considered as potential human pathogens (M. avium-complex [BA 12, BA 31] and M. scrofulaceum [BA 24]) induced minor inflammatory and cytotoxic responses compared to the nonpathogenic M. terrae [BA 20, BA 26]. Consistently with our findings, it has been reported previously that virulence of M. avium is inversely proportional to production of important inflammatory mediator TNF-α in mouse macrophages (Sarmento and Appelberg, 1995).

In conclusion, these results indicate that exposure to environmental mycobacteria, isolated from moldy houses, initiates a cascade of events leading to production of immunostimulatory and reactive compounds, and eventually cell death in murine RAW264.7 macrophages. If this is the case also in vivo, environmental mycobacteria are an important group of microbes, which may contribute to the adverse health effects observed among the inhabitants of the buildings with moisture and mold damage.
Acknowledgements

The authors wish to thank Heli Martikainen, Virpi Koponen and Leena Heikkilä for their excellent technical assistance. This study was supported by the Finnish Work Environment Fund and the Academy of Finland.

References


COMPARISON OF MYCOBACTERIA-INDUCED CYTOTOXICITY AND INFLAMMATORY RESPONSES IN HUMAN AND MOUSE CELL LINES

Kati Huttunen, Juha Jussila, Maija-Riitta Hirvonen
Laboratory of Toxicology, National Public Health Institute, Kuopio, Finland

Eila Iivanainen
Laboratory of Environmental Microbiology, National Public Health Institute, Kuopio, Finland

Marja-Leena Katila
Department of Clinical Microbiology, Kuopio University Hospital, Kuopio, Finland

Environmental mycobacteria, which are ubiquitous in nature, are also detected in moisture-damaged buildings. Their potential role inducing the adverse health effects associated with living in moisture damaged buildings requires clarification. To establish a model for these studies, we evaluated inflammatory responsiveness in different cell lines exposed to environmental mycobacterial species. Four mycobacterial isolates belonging to Mycobacterium avium complex and Mycobacterium terrae, recovered from the indoor air sampled when a moldy building was being demolished, were studied for their cytotoxicity and ability to stimulate the production of inflammatory mediators in mouse RAW264.7 and human 285C macrophage cell lines, and human A549 lung epithelial cell line. Lipopolysaccharide (LPS) was used as a positive control. Production of cytokines (tumor necrosis factor α, TNF-α; interleukin 6, IL-6; and interleukin β, IL-1β) was analyzed immunochemically, nitric oxide (NO) by the Griess method, expression of inducible NO synthase with Western blot analysis, and cytotoxicity with the MTT test. Both human and mouse cells produced NO and IL-6 after mycobacterial exposure. Mouse macrophages also showed production of TNF-α induced by both mycobacteria and LPS, whereas the human cell lines failed to produce TNF-α after mycobacterial exposure and the human epithelial cell line also failed to respond to LPS. Similarly, only mouse macrophages produced IL-1β. Mycobacterial exposure was not cytotoxic to human cells and was only slightly cytotoxic to mouse macrophages. The results indicate that environmental mycobacterial isolates from moldy buildings are capable of activating inflammatory mechanisms in both human and murine cells. The human and mouse cell lines, however, differ significantly in the grade and type of the responses.

Received 12 May 2001; sent for revision 5 June 2001; revision received 23 June 2001; accepted 29 June 2001.

The authors thank Heli Martikainen and Leena Heikkinen for their excellent technical assistance. This study was supported by the Academy of Finland, the Finnish Research Programme on Environmental Health, and Farmers’ Social Insurance Institution.

Address correspondence to Kati Huttunen, Division of Environmental Health, National Public Health Institute, PO Box 95, FIN-70701 Kuopio, Finland. E-mail: Kati.Huttunen@ki.fi
There is accumulating epidemiological evidence pointing to an association between microbial growth in moisture-damaged buildings and clinical symptoms among the inhabitants (Brunekreef, 1992; Spengler et al., 1994; Taskinen et al., 1997). This view is supported by our earlier in vitro data demonstrating how streptomycetes, isolated from a building suffering moisture and mold damage, are capable of inducing the production of inflammatory mediators in cell lines (Hirvonen et al., 1997; Jussila et al., 1999). Consistent with these results, markers of acute inflammation have also been observed in vivo in mice after exposure to *Streptomyces californicus* (Jussila et al., 2001). Moreover, we have recently shown that exposure to environmental mycobacteria can activate the production of inflammatory mediators in murine macrophages (Huttunen et al., 2000).

In summary, this murine data suggests that the inflammatory response to certain microbial components is a plausible factor contributing to the reported adverse health effects suffered by inhabitants of moisture and mold damaged buildings. It is not known whether similar responses to mycobacteria occur also in human cells. The inflammatory potency of different species or strains of mycobacteria may also vary.

The proinflammatory cytokines, tumor necrosis factor alpha (TNF-α) and interleukin 1β (IL-1β), are known to stimulate the production of interleukin 6 (IL-6), which is mainly responsible for the acute-phase response (Van Snick, 1990). According to our earlier experimental data obtained with a mouse macrophage cell line (RAW264.7) exposed to mycobacterial isolates, the increase in TNF-α production precedes the IL-6-response, which we interpret as suggestion of a causal relationship (Huttunen et al., 2000).

Nitric oxide (NO) is an important mediator of microbicidal activity in murine macrophages, though its production and effector role in human cells is less certain. Although several studies indicate that human macrophages can produce small amounts of NO and express the inducible form of nitric oxide synthase (iNOS) after exposure to different stimuli (Rich et al., 1997; Jagannath et al., 1998; Rockett et al., 1998; Boomershine et al., 1999; Brighbill et al., 1999), there are also contradictory reports (Arias et al., 1997; Fazal, 1996). It has recently been demonstrated that iNOS is expressed in human tissues containing granulomas associated with intracellular pathogens, including mycobacteria (Facchetti et al., 1999). Thus expression of iNOS may be induced in human monocytes and monocyte-derived cells in infections and chronic inflammatory diseases. It still remains to be confirmed to which degree the antimicrobial activity of the human macrophages is in fact dependent on nitric oxide production resulting from iNOS expression.

We hypothesize that the inflammatory responses of human and murine cells to mycobacterial exposure have similarities, which can be utilized in the extrapolation from the murine data to human exposure. The aim of the present study was to compare two human cell lines with a mouse macrophage cell line for their inflammatory and cytotoxic responsiveness.
to exposure to environmental mycobacteria. The responses were followed time- and dose-dependently, to permit evaluation of which of the measured inflammatory markers could be best suited when studying ongoing responses in human and mouse cell lines.

**METHOD**

**Mycobacteria**

Four mycobacterial isolates were recovered from the indoor air of a moldy building during demolition work. For identification to species level, the isolates were analyzed for cellular fatty acid and alcohol composition using gas–liquid chromatography (GLC) (Torkko et al., 1998). In addition, the isolates were tested for growth and biochemical characteristics, and for hybridization with commercial DNA probes (AccuProbe; GenProbe, Inc., San Diego, CA) as described previously (Torkko et al., 1999). The isolates belonged to two species; a potentially pathogenic Mycobacterium avium-complex (isolate BA 12; MAC 1 and isolate BÀ31; MAC 2) and a nonpathogenic species of Mycobacterium terrae (isolate BA20; M. terrae 1 and isolate BA26; M. terrae 2). For the present study, the mycobacterial isolates were cultured on Mycobacteria 7H11-agar supplemented with OADC-enrichment (Difco Laboratories, Detroit, Michigan, USA) at 30°C. After four weeks, the bacterial cells were harvested, washed, suspended in Hank’s Balanced Salt Solution (HBSS) (Gibco BRL, Paisley, UK) and sonicated for 15 min. Concentrations were counted at 1250 × magnification using an epifluorescence microscope (Olympus BH2-RFL, Olympus Optical Co., Tokyo, Japan) and acridine orange staining modified for mycobacteria (Hobbie et al., 1977; Katila et al., 1982).

**Cell Culture**

A mouse macrophage cell line (RAW264.7), a human macrophage cell line (28SC) and a human lung epithelial cell line (A549) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). All the cell types were cultured at 37°C in 5% CO₂ atmosphere in cell line specific culture medium.

Mouse RAW264.7 macrophages were cultured in RPMI 1640-medium supplemented with 10% of heat inactivated fetal bovine serum (FBS), 1% of 1-glutamine and 1% of Penicillin-Streptomycin (all from Gibco BRL, Paisley, UK). The human 28SC macrophages (hematopoietic cell line) were cultured in Iscove’s modified Dulbecco’s medium supplemented with 10% of heat inactivated FBS, 0.05 mM 2-mercaptoethanol, 0.03 mM thymidine and 0.01 mM hypoxanthine. Human alveolar type II epithelial-like cell line A549 was cultured in Ham’s F-12K Medium supplemented with 10% heat inactivated FBS. Cells (5 × 10⁵ cells/ml) were dispensed to 6-well plates, 2 ml/well. The human cell lines were primed with INF-γ (10 ng/
ml) and anti-microbial agents (nystatin and penicillin-streptomycin) were added after the dispensing of the cells. The cells were allowed to adhere for 24 h and, before exposure, fresh complete medium was added. The human macrophage cells grow in suspension and hence were exposed without changing the culture medium, also 24 h after the dispersion.

**Experimental Design**

In the dose-response study, the human cell lines (28SC and A549) were exposed to 3 doses (10^5, 10^6, and 10^7 mycobacterial cells/ml) of each of the 4 strains of mycobacteria for 24 h. For the time-course study, all the 3 cell lines were exposed to all 4 isolates of mycobacteria at the dose of 10^7 mycobacterial cells/ml, and the exposure was terminated at 5 time points (3, 6, 12, 24, and 48 h). The dose was chosen on the basis of the dose response results of the human cell lines in the current study and our earlier studies with mouse RAW264.7 macrophages (Huttunen et al., 2000). After the exposure, the adherent cells were resuspended in the culture medium either by scraping (RAW264.7) or trypsin incubation (A549), and the cell suspension was centrifuged (5 min, 8000 rpm) in order to separate the cells from the culture medium. The supernatants were stored at −80°C for the analyses of cytokines, and the cells were frozen immediately in dry ice and stored at −80°C for Western blot analysis.

**Nitrite Analysis**

Nitric oxide was measured spectrophotometrically as the stable metabolite, nitrite (NO₂⁻) according to the Griess method (Green et al., 1982). Briefly, Griess reagent (1% sulfanilamide and 0.1% naphthylethylene-diamine dihydrochloride in 2% phosphoric acid) was mixed 1:1 with samples of the cell culture medium. Nitrite forms a colored chromophore with the reagent, with an absorbance maximum at wavelength of 543 nm, which was measured with an enzyme-linked immunosorbent assay (ELISA) microplate reader (iEMS Reader MF, Labsystems, Turku, Finland). The production of nitrite was quantified by comparing the results with absorbances of standard solutions of sodium nitrite (NaNO₂). Samples of three independent experiments were analyzed in duplicate.

**Western Blot Analysis**

Expression of the iNOS was analyzed with the Western blot technique. The frozen cells were disintegrated in lysis buffer [20 mM TrisHCl, 2 mM ethylenediamine tetraacetic acid (EDTA), 3% (v/v) Triton X-100, 100 mM NaCl] by pulling the cell suspension through a 26G needle and incubating the suspension for 30 min on ice. Afterward the cell fragments were centrifuged (13,000 rpm) for 10 min. Sample buffer (1.5 mM bromophenol blue; 0.14 M sodium dodecyl sulfonate (SDS); 0.12 M TrisHCl; 0.1 M β-mercaptoethanol; 1.3 M glycerol) was heated and added 1:2 to the supernatant, which was boiled at 95°C for 6 min. Samples were subjected to elec-
trophoresis through 7.5% Tris-Glycine Gel (Criterion gels, Bio-Rad Laboratories, Hercules, CA) in order to differentiate proteins by size. From the gel, proteins were transferred electrophoretically in a transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol, 0.05% SDS) to a PVDF membrane.

Membranes were incubated overnight at 4°C in blocking buffer [100 mM TrisHCl; 150 mM NaCl; 50 g/L bovine serum albumin (BSA)], rinsed with washing buffer (10 mM TrisHCl; 150 mM NaCl; 0.1% Tween; pH 7.4) and incubated in primary antibody solution (0.1% rabbit anti-iNOS polyclonal antibody (Transduction Laboratories, USA) in washing buffer) for 2 h at room temperature. After the primary incubation, the membranes were washed (6 x 5 min) with washing buffer and incubated in a second antibody solution [0.05% alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG, Zymax Zymed, CA) in washing buffer] for 1 h. The membranes were washed again, bathed in developing buffer (100 mM NaCl; 100 mM Tris; 50 mM MgCl•6H2O) and developed in developing solution [330 mg/L nitro blue tetrazolium (NBT); 165 mg/L bromochloroindolyl phosphate disodium (BCIP) (both from Sigma Chemical Co., MO) in developing buffer]. Colored bands of the size of 130 kD were visually detected from the membrane.

**Cytokine Analysis**

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

**Cell Viability**

Viability of the macrophages was determined by using the 3-(4,5-di- methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) test to detect functioning mitochondria (Mosmann, 1983). Live mitochondria can transform MTT (Sigma Chemical Co., St. Louis, MO) to formazan, which can be measured with a spectrophotometer. The proportion of viable cells in exposed samples was compared to control samples. In addition, the viability of the cells in the control samples was estimated after staining the cells with trypan blue solution. Samples of three independent experiments were analyzed in duplicate.

**Statistical Analysis**

The data were statistically analyzed using analysis of variance (ANOVA) and a least significant difference (LSD) test to compare the exposed group to the control group (SPSS, version 7.51, SPSS Inc., 1996). The difference was considered to be statistically significant at p < .05.
RESULTS

The Production of TNF-α

The human cell lines 28SC and A549 did not produce TNF-α in response to the exposure with the tested mycobacterial isolates at any dose (Table 1) or time point (Figure 1). The human 28SC macrophages produced significant amounts (94 ± 56 pg/ml at 3 h) of TNF-α when exposed to 10 μg/ml LPS, whereas no response to LPS was detected in the human epithelial A549 cells (Figure 2). In contrast to the human cells, mouse RAW264.7 macrophages produced large amounts of TNF-α after exposure to both of the mycobacterial isolates used in these analyses (Figure 1) and LPS (Figure 2). All four mycobacterial isolates caused a similar

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Exposure agent</th>
<th>Dose (mycobacterial cells/ml)</th>
<th>Nitrite (μM)</th>
<th>Cell viability (% of control)</th>
<th>IL-1β (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human 28SC</td>
<td>LPS</td>
<td>10 μg/ml</td>
<td>2.1 ± 0.6</td>
<td>72 ± 9</td>
<td>74.5 ± 0.7</td>
<td>580 ± 40</td>
<td>BD</td>
</tr>
<tr>
<td>M. avium complex (MAC 1)</td>
<td>LPS</td>
<td>10 μg/ml</td>
<td>1.5 ± 0.2</td>
<td>94 ± 5</td>
<td>BD</td>
<td>BD</td>
<td>BD</td>
</tr>
<tr>
<td>M. terræ 1</td>
<td>LPS</td>
<td>10 μg/ml</td>
<td>1.6 ± 0.3</td>
<td>96 ± 7</td>
<td>BD</td>
<td>BD</td>
<td>BD</td>
</tr>
<tr>
<td>M. terræ 2</td>
<td>LPS</td>
<td>10 μg/ml</td>
<td>2.0 ± 0.3</td>
<td>95 ± 7</td>
<td>BD</td>
<td>BD</td>
<td>BD</td>
</tr>
<tr>
<td>Human A549</td>
<td>LPS</td>
<td>10 μg/ml</td>
<td>1.5 ± 0.1</td>
<td>96 ± 5</td>
<td>BD</td>
<td>BD</td>
<td>BD</td>
</tr>
<tr>
<td>M. avium complex (MAC 1)</td>
<td>LPS</td>
<td>10 μg/ml</td>
<td>1.8 ± 0.4</td>
<td>100 ± 4</td>
<td>BD</td>
<td>BD</td>
<td>BD</td>
</tr>
<tr>
<td>M. terræ 1</td>
<td>LPS</td>
<td>10 μg/ml</td>
<td>2.3 ± 0.3</td>
<td>102 ± 3</td>
<td>BD</td>
<td>3.6 ± 2.1</td>
<td>BD</td>
</tr>
<tr>
<td>M. terræ 2</td>
<td>LPS</td>
<td>10 μg/ml</td>
<td>1.7 ± 0.2</td>
<td>95 ± 6</td>
<td>BD</td>
<td>BD</td>
<td>BD</td>
</tr>
</tbody>
</table>
**Tumor necrosis factor alpha**

**Mouse RAW264.7 macrophages**

**Human 28SC macrophages**

**Human A549 lung epithelial cells**

**Interleukin-1**

**Mouse RAW264.7 macrophages**

**Human 28SC macrophages**

**Human A549 lung epithelial cells**

**Interleukin-6**

**Mouse RAW264.7 macrophages**

**Human 28SC macrophages**

**Human A549 lung epithelial cells**

□ Control □ MAC 1 □ MAC 2 □ M. terrae 1 □ M. terrae 2

**FIGURE 1.** Time course of TNF-α, IL-1, and IL-6 production in mouse RAW264.7 macrophages, human 28SC macrophages, and human A549 lung epithelial cells after exposure to a single dose (10⁷ mycobacterial cells/ml) of four mycobacterial isolates (Mycobacterium avium complex (MAC) 1 and 2; Mycobacterium terrae 1 and 2). Inset in IL-6 chart shows a smaller concentration range at the first time point. Note the different scale in induced production of cytokines in mouse and human cell lines. Each column represents mean ± SEM of three independent experiments done in duplicate. Star indicates statistically significant difference from control, *p < .05.*
Tumor necrosis factor alpha

**FIGURE 2.** Time course of TNF-α, IL-1, and IL-6 production in mouse RAW264.7 macrophages, human 28SC macrophages, and human A549 lung epithelial cells after exposure to a single dose (10 μg/ml) of lipopolysaccharide (LPS). Note the different scale in induced production of cytokines in mouse and human cell lines. Each column represents mean ± SEM of three independent experiments done in duplicate. Star indicates statistically significant difference from control, p < .05.
response in mouse RAW264.7 macrophages, although the response to *M. avium* isolates was slightly more rapid than found for *M. terrae* (Figure 1). The highest responses were detected with the *M. avium* complex (isolate MAC 1), which induced a significant response (2900 ± 230 pg/ml) already at 3 h after the exposure, and the level further increased up to 24 h (3960 ± 340 pg/ml), gradually declining thereafter, though it was still as high as 3520 ± 460 pg/ml at 48 h after the exposure (Figure 1).

**The Production of IL-1β**

The human cell lines (28SC, A259) did not produce markedly increased amounts of IL-1β after exposure to LPS (Figure 2) or any tested mycobacterial isolates at any tested dose (Table 1) or time point (Figure 1) in this study. In contrast, mouse RAW264.7 macrophages produced increasing amounts of IL-1β in a time-dependent manner from the beginning of the mycobacterial exposure until 12 h (up to 160 ± 60 pg/ml). Thereafter the response declined, returning to the control levels at time points 24 and 48 h (Figure 1). Similarly, the LPS-induced IL-1β response peaked at 12 h, attaining values as high as 1930 ± 450 pg/ml (Figure 2). *Mycobacterium avium* complex was better than *M. terrae* at inducing IL-1β production in mouse cells (Figure 1).

**The Production of IL-6**

IL-6 production was increased in all three cell lines after exposure to mycobacteria. Human 28SC macrophages produced IL-6 after exposure to the dose of 10⁷ mycobacterial cells/ml of all of the studied mycobacterial strains (Table 1). The increase in IL-6 production was evident at 24 h after the exposure, whereas nonexposed control cells produced barely detectable amounts of this cytokine at all time points (0.7 ± 0.3 pg/ml at 48 h). The production of IL-6 increased in a time-dependent manner, reaching 7.5 ± 2 pg/ml at 48 h (Figure 1).

Human A549 epithelial cells were also induced to produce IL-6, but the baseline level of this cytokine was higher in these cells than in human macrophages (15.0 ± 0.5 pg/ml at 48 h). However, a clear difference compared to control cells could be detected at 48 h, the most potent inducer being *M. avium* complex (isolate MAC 1: 23 ± 2 pg/ml).

The highest IL-6 response was detected in mouse RAW264.7 macrophages, which produced significant amounts of this cytokine in a time-dependent manner already after 6 h of exposure. In RAW264.7 cells, IL-6 production induced by *M. avium* complex reached its maximum (isolate MAC 1: 2230 ± 30 pg/ml) at 24 h after the exposure, whereas the *M. terrae*-induced IL-6 production was highest (isolate *M. terrae* 1; 1820 ± 220 pg/ml) at the end of the follow-up, 48 h after the exposure (Figure 1).

**The Production of NO**

Exposure to mycobacteria evoked NO production, assessed as nitrite in cell culture medium, in all three cell lines. In human cells, only the highest
dose (10⁷ mycobacterial cells/ml) induced a slight increase in the production of nitrite (Table 1). In the time-course study, both apathogenic *M. terrae* isolates (*M. terrae* 1 and *M. terrae* 2) were more potent than the *M. avium* complex. The time-dependent NO production increased up 48 h, reaching 2.7 ± 0.2 μM in human 28SC macrophages and 2.1 ± 0.6 μM in human A549 lung epithelial cells (Figure 3A). However, the background production of NO in control cells was relatively high (1.7 ± 0.2 μM and 1.7 ± 0.5 μM, respectively) (Figure 3A). Mouse RAW264.7 macrophages produced significant amounts of NO already at 3 h from the beginning of the expo-

**Nitric oxide**

![Graph showing nitric oxide production](image)

**FIGURE 3.** (A) Time course of nitrite production after exposure to single doses (10⁷ mycobacterial cells/ml) of four mycobacterial isolates (*Mycobacterium avium* complex [MAC] 1 and 2; *Mycobacterium terrae* 1 and 2) and 10 μg/ml of lipopolysaccharide (LPS) in mouse RAW264.7 macrophages, human 28SC macrophages, and human A549 lung epithelial cells. Inset in chart shows a smaller concentration range at the first two time points. Note the different scale in the induced response in mouse and human cell lines. Each column represents mean ± SEM of three independent experiments done in duplicate. Star indicates statistically significant difference from control, *p* < .05. (B) Time course of iNOS expression in RAW264.7 macrophages after the exposure to *Mycobacterium terrae* isolate 2, shown for a representative sample.
Cell viability

**FIGURE 4.** Decrease in cell viability induced by exposure to single doses (10⁷ mycobacterial cells/ml) of four mycobacterial isolates [Mycobacterium avium complex (MAC) 1 and 2; Mycobacterium terrae 1 and 2] in mouse RAW264.7 macrophages, human 28SC macrophages, and human A549 lung epithelial cells during 48-h period. Each symbol represents mean ± SEM of three independent experiments done in duplicate. Star indicates statistically significant difference from control, p < .05.

sure, and NO production further increased until the end of the follow-up (48 h), reaching a level as high as 34.5 ± 9.1 μM (Figure 3A). In contrast to human cell lines, M. avium complex (isolates MAC 1 and MAC 2) was more potent than the M. terrae isolates. iNOS was detected from the mouse RAW264.7 macrophages concomitantly with markedly increased nitrite levels in the cell culture medium (Figure 3B). No expression of iNOS was detected in human cells with Western blot analysis.

**Cell Viability**

Mycobacterial or LPS exposure was not markedly cytotoxic to human A549 cell lines at any tested dose or time point, whereas the viability of the human 28SC macrophages slightly declined after exposure to 10 μg/ml of LPS (up to 27 ± 8% dead cells compared to control cells at 48 h) (Table 1 and Figure 4). The viability of the mouse RAW264.7 macrophages was time-dependently deteriorated by mycobacteria and especially by LPS (down to 14 ± 3% viable cells compared to control at 48 h). Of the tested mycobacterial isolates, exposure to M. avium complex (isolate MAC 1) caused the greatest cytotoxic effect in the mouse cells (60 ± 8% viable cells) compared to control cells at 48 h (Figure 4).
DISCUSSION AND CONCLUSIONS

This data clearly indicate that both human and mouse cells are capable of responding to exposure to environmental mycobacteria by producing inflammatory markers. There were, however, distinct differences in the extent and type of induced responses between human macrophages and lung epithelial cells as well as between human and mouse macrophages. In general, the levels of induced responses to these microbes were significantly lower in human cells than in mouse cells. There were also differences between human and mouse cells in terms of cytotoxicity and in the spectrum of cytokines produced by exposure to both mycobacteria and LPS.

IL-6 production was the most consistent indicator of inflammatory responses in all of the cell lines. The production of IL-6 increased in a time-dependent manner in all three of the cell lines in response to mycobacteria and LPS. There was, however, a quantitative difference in the responses measured. The concentrations achieved in the mouse cell line were a hundredfold higher than the corresponding concentrations in the human cell lines. Interestingly, the production of TNF-α, a reproducible and rapid indicator of inflammation in mouse macrophages, proved to be of little importance in the human cells. Only LPS induced human 28SC macrophages to produce TNF-α, whereas the human A549 lung epithelial cells never produced TNF-α. This finding is consistent with earlier published data, which have also shown a failure of LPS to stimulate production of TNF-α in a human lung epithelial A549 cell line (Wang et al., 1999). Similarly, the mouse RAW264.7 cell line was the only one to produce significant amounts of proinflammatory cytokine IL-1β at the studied time points. The kinetic properties of this cytokine differ from the other cytokines measured. The IL-1β production seems to fluctuate in a rather fast time frame, which complicates the definition of the time point of maximum or minimum production. Since the human cells did not produce significant amounts of IL-1β during this experiment, the IL-1β level in cell culture medium is a rather poor marker for the inflammatory responses in these cell lines. The current data demonstrate clearly that neither TNF-α nor IL-1β is required to trigger the production of IL-6 in these cell lines, although these agents are known to stimulate IL-6 production via autocrine and paracrine pathways (Van Snick, 1990).

The data presented here revealed how NO production could be induced in human and mouse macrophages by mycobacteria and LPS. However, the amount of NO produced by human 28SC macrophages and human A549 lung epithelial cells was low, and external conditions and artifacts often interfered with its determination. The concentrations detected were at the lower limit of detection by the Griess method. This limitation was also noted by Jagannath and coworkers when comparing colorimetric and fluorometric methods for the detection of NO produced by human monocyte/macrophages (Jagannath, 1998). In our experimental settings, no iNOS expression was detected in the human cell lines by Western blotting,
whereas the mouse macrophages showed a distinct expression of iNOS. However, the human lung epithelial cells have been reported to express iNOS mRNA after stimulation with supernatant fluids of peripheral blood mononuclear cells cocultured with tubercle bacilli (Kwon et al., 1998). Earlier studies indicate that iNOS expression can be detected in alveolar macrophages obtained from patients infected with M. tuberculosis (Wang et al., 1998). It seems clear that the in vitro production of NO derivatives and expression of iNOS in activated human monocytes still need further investigation. The presence of activating factors such as interferon gamma (IFN-γ) and leukocytes (Rockett et al., 1998; Bonecini-Almeida et al., 1998) and also the increased sensitivity of the methods used in detection (Jagannath et al., 1998) have improved the applicability of in vitro human monocyte/macrophage models.

Consistent with earlier findings, the studied mycobacterial isolates were only moderately cytotoxic to mouse macrophages, whereas LPS markedly decreased cell viability. This implies that the effect of exposure to mycobacteria is not a radical destruction of the cell but rather interference in the normal cell cycle. The mycobacterial exposure was nontoxic to both human cell lines at every time point and with all doses of mycobacteria used. Moreover, LPS was not cytotoxic to human A549 lung epithelial cells and was only moderately cytotoxic to human 28SC macrophages, in contrast to its ability to cause a significant time-dependent decrease in cell viability in mouse RAW264.7 macrophages.

The complex cell wall of mycobacteria contains lipoarabinomannan (LAM), which is known to possess substantial immunostimulatory properties. Interestingly, structural differences in the LAM of various mycobacterial strains have been observed to influence the immunoresponsiveness and possibly also the virulence of the strains (Roach et al., 1993; Sarmento & Appelberg, 1995).

In conclusion, our results indicate that environmental mycobacterial isolates from moldy buildings are capable of activating inflammatory mechanisms in both human and murine cells. The human and mouse cell lines, however, differ significantly in the grade and type of the responses. The selection of cell lines and inflammatory mediators to be used in analyses needs to be carefully assessed when evaluating inflammatory or cytotoxic potentials of mycobacteria. Comparison of data obtained from studies using different cell lines provides valuable basic information that is necessary also for risk assessment when extrapolating the results of in vitro and in vivo models to human exposures.

REFERENCES


Reproduced with permission from Environmental Health Perspectives.
Production of Proinflammatory Mediators by Indoor Air Bacteria and Fungal Spores in Mouse and Human Cell Lines

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

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

We compared the inflammatory and cytotoxic responses caused by household mold and bacteria in human and mouse cell lines. We studied the fungi Aspergillus versicolor, Penicillium spinulosum, and Stachybotrys chartarum and the bacteria Bacillus cereus, Pseudomonas fluorescens, and Streptomyces californicus for their cytotoxicity and ability to stimulate the production of inflammatory mediators in mouse RAW264.7 and human 293C macrophage cell lines and in the human A549 lung epithelial cell line in 24-h exposure to 10^6, 10^7, and 10^8 CFUs/mL. We studied time dependency by terminating the exposure to 10^6 CFUs/mL after 3, 6, 12, 24, and 48 h. We analyzed production of the cytokines tumor necrosis factor-α and interleukins 6 and 10 (TNF-α, IL-6, IL-10, respectively) and measured nitric oxide production using the Griess method, expression of inducible NO-synthase with Western blot analysis, and cytotoxicity with the MTT-test. All bacteria strongly induced the production of TNF-α, IL-6 and, to a lesser extent, the formation of IL-10 in mouse macrophages. Only the spores of Streptomyces californicus induced the production of NO and IL-6 in both human and mouse cells. In contrast, exposure to fungal strains did not markedly increase the production of NO or cytokine in the studied cell lines except for Stachybotrys chartarum, which increased IL-6 production somewhat in human lung epithelial cells. These microorganisms were less cytotoxic to human cells than to mouse cells. On the basis of equivalent numbers of bacteria and spores of fungi added to cell cultures, the overall potency to stimulate the production of proinflammatory mediators decreased in the order Ps. fluorescens > Streptomyces californicus > B. cereus > Sta. chartarum > A. versicolor > P. spinulosum. These data suggest that bacteria in water-damaged buildings should also be considered as causative agents of adverse inflammatory effects. Key words: bacteria, cytokine production, fungi, inflammation, mold.


doi:10.1289/ehp.5478 available via http://dx.doi.org/

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

Indoor bacterial flora include both gram-positive and gram-negative bacteria, which grow in moist building materials (Anderson et al. 1997; Hyvärinen et al. 2002). The gram-positive sporing bacteria Streptomyces spp. have been frequently isolated from water-damaged buildings, but they are not part of the normal microbial flora of indoor air (Nevalainen et al. 1991). The presence of Streptomyces is also an indicator of moisture damage in buildings (Samson et al. 1994). We demonstrated previously that the spores of streptomycetes originating from moldy buildings can evoke intense production of inflammatory mediators both in mouse and human cell lines (Hirvonen et al. 1997; Jussila et al. 1999) in vitro and in mouse lungs in vivo (Jussila et al. 2001). Thus, the correlation found between in vitro and in vivo data strongly supports the view that streptomycetes play a role in the cascade of events leading to adverse health effects in occupants of moldy buildings. Moreover, these data suggest that phagocytes and epithelial cells may be the target cells producing these inflammatory mediators in the lung.

The gram-positive bacteria Bacillus spp. are common environmental bacteria and are also found in indoor environments. Recently, Bacillus cereus has been isolated from damaged building materials, and also toxin-producing strains of Bacillus spp. have been identified (Anderson et al. 1999). However, the ability of these strains to cause inflammatory responses has not been shown. In contrast to gram-positive bacteria, all gram-negative bacteria contain lipopolysaccharide (LPS) as a component of their cell membrane. LPS has profound stimulating effects on immunological cells, inducing production of inflammatory mediators and nitric oxide (Raetz et al. 1991). Among the gram-negative bacteria, the genus Pseudomonas spp. is found in many natural sources, including soils, waters, and outdoor air. Ps. fluorescens is the most common Pseudomonas species in the outdoor air (Nevalainen 1989).

Penicillium and Aspergillus spp. are frequently detected at higher concentrations in moldy buildings, although these microbes are common airborne fungi in indoor environments (Flannigan and Mory 1996; Hyvärinen et al. 1995; Nevalainen et al. 1991). Both these genera include also mycotoxin-producing species (Frisvad and Graveven 1994), and thus exposure to these species can potentially lead to adverse health effects. However, the conditions supporting the toxin production and the mechanisms of exposure and the health effects have yet to be identified. Penicillium spinulosum is considered a generally nontoxic species, whereas Aspergillus versicolor is both a common toxin producer and an indicator of mold problems in a building (Samson et al. 1994). Stachybotrys chartarum, a well-known producer of potent toxins (Graveven et al. 1994), prefers a substrate conditioned with a high moisture content, thus indicating severe moisture damage when isolated from buildings (Johanning et al. 1996). The growth of this fungus in water-damaged buildings has been associated with adverse health effects such as respiratory and other symptoms (Gordon et al. 1999; Johanning et al. 1996, 1999). Moreover, Sta. chartarum has been associated with a cluster of cases of idiopathic pulmonary hemorrhage (IPH) in infants in North America and Europe (Centers for Disease Control and Prevention 1994; Hoppus et al. 1999).

Address correspondence to K. Huttunen, Department of Environmental Health, National Public Health Institute, PO Box 95, FIN-70701 Kuopio, Finland, Telephone: +358 14 203120, Fax: +358 14 201265, E-mail: Kati.Huttunen@ki.fi

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

Received 18 January 2002; accepted 18 June 2002.
Our aim in this study was to compare the cytotoxic and inflammatory potential of the fungal and bacterial strains considered important in indoor environments. We selected Aspergillus versicolor and Stachybotrys chartarum because of their characteristic occurrence in water-damaged buildings and their proposed inflammatory potential. We selected Bacillus cereus to represent another gram-positive bacteria and Penicillium aurantiogriseum because of its characteristic occurrence in water-damaged buildings and its proposed inflammatory potential. We selected Aspergillus versicolor, Stachybotrys chartarum, and Penicillium aurantiogriseum because of its characteristic occurrence in water-damaged buildings and its proposed inflammatory potential. We selected Aspergillus versicolor, Penicillium aurantiogriseum, and Bacillus cereus to represent a gram-negative bacteria. All the strains were cultured at 37°C in a 5% CO2 atmosphere in cell line-specific culture medium.

**Materials and Methods**

**Microbes.** In this study we used three fungal strains, Aspergillus versicolor, Penicillium aurantiogriseum, and Stachybotrys chartarum, and three bacterial strains, Bacillus cereus, Pseudomonas fluorescens, and Streptomyces cattleyae. The strains of Aspergillus versicolor and Penicillium aurantiogriseum were isolated from indoor air of buildings with moisture problems, and Stachybotrys chartarum was isolated from a damaged building material sample. Bacillus cereus and Pseudomonas fluorescens were isolated from indoor air of residence without proven water damage, and Streptomyces cattleyae was isolated from a building with moisture problems. Microbes were sampled from indoor air using a six-stage impactor and 2% malt extract agar (MEA; Biorack Diagnostics, Beuvais, France) for fungi and tryptone yeast-ghoce agar (TYG; Bacto Plate Count Agar, Difco Laboratories, Detroit, MI, USA) for bacteria. Stachybotrys chartarum was isolated on malt extract agar.

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

**Cell culture.** A mouse macrophage cell line (RAW264.7), a human macrophage cell line (ZNC), and a human lung epithelial cell line (A549) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). All the cell types were cultured at 37°C in 5% CO2 atmosphere in cell line-specific culture medium.

A mouse macrophage RAW264.7 macrophages (homoplastic cell line) were cultured in Iscove’s modified Dulbecco’s medium supplemented with 10% heat-inactivated FBS, 0.05 mM 2-mercaptoethanol, 0.05 mM thymidine, and 0.01 mM hypoxanthine. Human alveolar type II epithelial-like cell line A549 was cultured in Ham’s F-12K Medium supplemented with 10% heat-inactivated FBS. Cells (5 x 104 cells/ml) were dispensed to six-well plates, 2 ml/well.

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

In the dose-response study, the macrophages and epithelial cells were exposed to three doses (10³, 10⁴, and 10⁵ microbes/ml) of each microbe for 24 hr. For the time-course study, all three cell lines were exposed to all six microbial strains at the dose of 10⁵ microbes/ml, and the exposure was terminated at five time points after the start of exposure (3, 6, 12, 24, and 48 hr). The dose used in time course study was chosen on the basis of the responses seen in dose-response study. We used bacterial LPS as a positive control at the dose of 10 μg/ml. After the exposure, the adherent cells were resuspended in the culture medium either by scraping (RAW264.7) or trypsin incubation (A549), and the cell suspension was centrifuged (5 min, 8000 rpm) to separate the cells from the culture medium. The supernatants were stored at -80°C for the analyses of cytokines, and the cells were frozen immediately in liquid nitrogen and stored at -80°C for Western blot analysis.

**Nitrile analysis.** We measured nitrile oxide using a spectrophotometrically as the stable metabolite, nitrite (NO₂) according to the Griess method (Green et al. 1982). Briefly, Griess reagent (1% sulfanilamide and 0.1% naphthalhydrazide dihydrochloride in 2% phosphoric acid) was mixed 1:1 with samples of the cell culture medium. Nitrite forms a colored chromophore with the reagent, with an absorbance maximum at a wavelength of 543 nm, which was measured with an enzyme-linked immunosorbent assay (ELISA) microplate reader (EMG Reader M1; Lantensys, Turku, Finland). We quantified the production of nitrite by comparing the results with absorbances of standard

![Figure 1](https://example.com/image1.png)  
**Figure 1.** The time frame of TNF-α production after exposure to a single dose (10³ microbes/ml) of A. versicolor (ASP), P. aurantiogriseum (PEN), S. chartarum (STA), B. cereus (B), P. fluorescens (STF), and A mouse RAW264.7 macrophages, (B) human A549 cells, and (C) human A549 lung epithelial cells. Each column represents mean ± SEM of three independent experiments done in duplicate.  
*Statistically significant difference from the respective control, p < 0.05.*
solutions of sodium nitrite. Samples of three independent experiments were analyzed in duplicate.

Western blot analysis. We analyzed expression of the inducible nitric oxide synthase (iNOS) with the Western blot technique. The frozen cells were lyzed in lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 5% [v/v] Triton X-100, 100 mM NaCl) by pulling the cell fragments centrifuged (13,000 rpm) for 10 min. Sample buffer (1.5 mM bromophenol blue, 0.14 M SDS; 0.12 M Tris-HCl, 0.1 M β-mercaptoethanol; 1.5 M glycerol) was heated and added 1:2 to the supernatant, which was subsequently boiled at 95°C for 6 min. The samples were subjected to elec-
trophoresis through 7.5% Tris-glycine gel (Criterion gels; Bio-Rad Laboratories, Hercules, CA, USA) to differentiate proteins by size. From the gel, proteins were trans-
ferred electrophoretically in a transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol, 0.05% SDS) to a polyvinyl difluo-
ride membrane.

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

the primary incubation, we washed (6 x 5 min) the membranes with washing buffer and incubated them in a secondary antibody solution (0.05% alkaline phosphatase conjugated goat anti-rabbit IgG (Zymark Zymed, South San Francisco, CA, USA) in washing buffer) for 1 hr. The membranes were washed again, baked in developing buffer (100 mM NaCl; 100 mM Tris; 50 mM MgCl2 x 6 H2O) and developed in developing solution [150 mg/L nitro blue tetrazolium (NBT); 165 mg/L bro-
moschelonitrile phosphate diisodium (BCIP; both from Sigma Chemical Company, St. Louis, MO, USA) in develop-
ing buffer]. Colored bands 130 kD in size were visually detected from the membrane.

Cytokine analysis. We analyzed TNF-α, IL-6, and IL-1β from the cell culture medium immunohistochemically using commer-
cial ELISA kits (B&K Systems, Minneapolis, MN, USA), as described earlier (Rooaalainen et al. 1998). Samples were processed accord-
ing to the manufacturer’s protocol and ana-
yzed with an ELISA microplate reader by comparing the absorbances of the samples to the
standard curve. We analyzed samples of three independent experiments in duplicate.

Cell viability. We determined the viability of the macrophages by using the 3-(4,5-
dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test to detect living mitochondria (Moumann 1983). Live mito-
chondria can transform MTT (Sigma) to for-
mazan, which can be measured with a spectrophotometer. We compared the propor-
tion of viable cells in exposed samples to a
control sample. The presence of microbes among the exposed cells does not interfere with the MTT test at tested concentrations. We estimated the viability of the cells in the control samples after staining the cells with try-
pan blue solution. Samples of three indepen-
dent experiments were analyzed in duplicate.

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

Results

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

Human 28S macrophages produced sig-
ificant amounts of TNF-α only after the exposure to LPS. This peaked at 3 hr (up to 90 ± 60 pg/mL) and gradually declined during the time course of the experiment. P. fluorescens caused a slight increase in the production of TNF-α, whereas the other microbes did not stimulate the human 28S macrophages to produce TNF-α (Table 2, Figure 1). Neither LPS nor any of the tested fungal or bacteria triggered TNF-α produc-
tion in human A349 epithelial cell line (Figure 1, Table 3).

Interleukin-6. Mouse RAW264.7 macro-
phages produced IL-6 in a dose- and time-
dependent manner after exposure to all three
bacterial species (R. eutropha, S. caenioceras, P. fluorescens, Table 1, Figure 2). The p-values for dose response were 0.006, 0.001, and 0.016, respectively. The positive control LPS also caused an intense IL-6 production (Table 1). Among the bacterial strains, the gram-negative P. fluorescens was the most potent. The spores of S. caenioceras also triggered a strong overall response in the mouse macrophages, whereas the induction of IL-6 by R. eutropha was clearly weaker (Table 1). The increase in the IL-6 production was detected at 3 hr of exposure, and it reached the maximum in 6 hr with P. fluorescens, whereas S. caenioceras displayed a slower time-course (Figure 2). The fungal strain A. versicolor, P. spiralis, and Sta. chartarum did not cause IL-6 production in mouse macrophages (Table 1).

### Table 2. Dose response of the production of inflammatory mediators and cell viability (mean ± SEM) of human 26B6 macrophages after 24 hr exposure to six microbes.

<table>
<thead>
<tr>
<th>Exposure agent</th>
<th>Dose (μg/mL)</th>
<th>NO (%)</th>
<th>Cell viability (% of control)</th>
<th>IL-1β (pg/mL)</th>
<th>TNF-α (pg/mL)</th>
<th>IL-6 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>—</td>
<td>100</td>
<td>BD</td>
<td>BD</td>
<td>BD</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>LPS</td>
<td>10 μg/mL</td>
<td>15 ± 0.2</td>
<td>106</td>
<td>82 ± 6*</td>
<td>BD</td>
<td>BD</td>
</tr>
<tr>
<td><em>fungi</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASP</td>
<td>10 ng/mL</td>
<td>15 ± 0.1</td>
<td>94 ± 6</td>
<td>BD</td>
<td>BD</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>10 ng/mL</td>
<td>15 ± 0.3</td>
<td>81 ± 3*</td>
<td>BD</td>
<td>BD</td>
<td>2 ± 1</td>
<td></td>
</tr>
<tr>
<td>PEN</td>
<td>10 ng/mL</td>
<td>15 ± 0.3</td>
<td>53 ± 6*</td>
<td>BD</td>
<td>BD</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>10 ng/mL</td>
<td>15 ± 0.2</td>
<td>76 ± 6*</td>
<td>BD</td>
<td>BD</td>
<td>1 ± 1</td>
<td></td>
</tr>
<tr>
<td>STA</td>
<td>10 ng/mL</td>
<td>15 ± 0.2</td>
<td>69 ± 6*</td>
<td>BD</td>
<td>BD</td>
<td>2 ± 0.3</td>
</tr>
<tr>
<td>10 ng/mL</td>
<td>15 ± 0.1</td>
<td>95 ± 6</td>
<td>BD</td>
<td>BD</td>
<td>1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>10 ng/mL</td>
<td>15 ± 0.2</td>
<td>36 ± 6*</td>
<td>BD</td>
<td>BD</td>
<td>3 ± 1</td>
<td></td>
</tr>
<tr>
<td><em>bacteria</em></td>
<td>10 ng/mL</td>
<td>15 ± 0.2</td>
<td>101 ± 4</td>
<td>BD</td>
<td>BD</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>BAC</td>
<td>10 ng/mL</td>
<td>15 ± 0.3</td>
<td>97 ± 4</td>
<td>BD</td>
<td>BD</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>10 ng/mL</td>
<td>15 ± 0.3</td>
<td>75 ± 6*</td>
<td>BD</td>
<td>BD</td>
<td>2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>STRE</td>
<td>10 ng/mL</td>
<td>15 ± 0.2</td>
<td>97 ± 6</td>
<td>BD</td>
<td>BD</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>10 ng/mL</td>
<td>15 ± 0.2</td>
<td>55 ± 6</td>
<td>BD</td>
<td>BD</td>
<td>14 ± 4</td>
<td></td>
</tr>
<tr>
<td>PSE</td>
<td>10 ng/mL</td>
<td>15 ± 0.2</td>
<td>75 ± 6*</td>
<td>BD</td>
<td>BD</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>10 ng/mL</td>
<td>15 ± 0.2</td>
<td>55 ± 6*</td>
<td>BD</td>
<td>BD</td>
<td>14 ± 4</td>
<td></td>
</tr>
<tr>
<td>*Abbreviations: ASP, Aspergillus versicolor; BAC, Bacillus caenioceras; BD, below detection limit; C, buffer control; NM, not measured; PEN, Penicillium spiralis; PSE, Pseudomonas fluorescens; STA, Stachybotrys chartarum; STRE, Streptomyces coelicolor. Statistical significance difference compared to buffer control, p &lt; 0.05.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Exposure agent</th>
<th>Dose (ng/mL)</th>
<th>NO (%)</th>
<th>Cell viability (% of control)</th>
<th>IL-1β (pg/mL)</th>
<th>TNF-α (pg/mL)</th>
<th>IL-6 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>—</td>
<td>100</td>
<td>BD</td>
<td>BD</td>
<td>BD</td>
<td>6 ± 0.6</td>
</tr>
<tr>
<td>LPS</td>
<td>10 μg/mL</td>
<td>15 ± 0.2</td>
<td>106</td>
<td>82 ± 6*</td>
<td>BD</td>
<td>BD</td>
</tr>
<tr>
<td><em>fungi</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASP</td>
<td>10 ng/mL</td>
<td>15 ± 0.3</td>
<td>103 ± 5</td>
<td>BD</td>
<td>BD</td>
<td>6 ± 0.5</td>
</tr>
<tr>
<td>10 ng/mL</td>
<td>15 ± 0.3</td>
<td>79 ± 5*</td>
<td>BD</td>
<td>BD</td>
<td>6 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>PEN</td>
<td>10 ng/mL</td>
<td>15 ± 0.3</td>
<td>80 ± 6</td>
<td>BD</td>
<td>BD</td>
<td>6 ± 0.8</td>
</tr>
<tr>
<td>10 ng/mL</td>
<td>15 ± 0.3</td>
<td>63 ± 6</td>
<td>BD</td>
<td>BD</td>
<td>14 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>STA</td>
<td>10 ng/mL</td>
<td>15 ± 0.2</td>
<td>125 ± 14</td>
<td>BD</td>
<td>BD</td>
<td>22 ± 5.5</td>
</tr>
<tr>
<td>10 ng/mL</td>
<td>15 ± 0.2</td>
<td>125 ± 14</td>
<td>BD</td>
<td>BD</td>
<td>22 ± 5.5</td>
<td></td>
</tr>
<tr>
<td><em>bacteria</em></td>
<td>10 ng/mL</td>
<td>15 ± 0.3</td>
<td>103 ± 5</td>
<td>BD</td>
<td>BD</td>
<td>6 ± 0.6</td>
</tr>
<tr>
<td>BAC</td>
<td>10 ng/mL</td>
<td>15 ± 0.3</td>
<td>103 ± 5</td>
<td>BD</td>
<td>BD</td>
<td>6 ± 0.6</td>
</tr>
<tr>
<td>10 ng/mL</td>
<td>15 ± 0.3</td>
<td>80 ± 6</td>
<td>BD</td>
<td>BD</td>
<td>14 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>STRE</td>
<td>10 ng/mL</td>
<td>15 ± 0.2</td>
<td>103 ± 12</td>
<td>BD</td>
<td>BD</td>
<td>22 ± 5.5</td>
</tr>
<tr>
<td>10 ng/mL</td>
<td>15 ± 0.2</td>
<td>103 ± 12</td>
<td>BD</td>
<td>BD</td>
<td>22 ± 5.5</td>
<td></td>
</tr>
<tr>
<td>PSE</td>
<td>10 ng/mL</td>
<td>15 ± 0.3</td>
<td>103 ± 5</td>
<td>BD</td>
<td>BD</td>
<td>6 ± 0.6</td>
</tr>
<tr>
<td>10 ng/mL</td>
<td>15 ± 0.3</td>
<td>80 ± 6</td>
<td>BD</td>
<td>BD</td>
<td>14 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>*Abbreviations: ASP, Aspergillus versicolor; BAC, Bacillus caenioceras; BD, below detection limit; C, buffer control; NM, not measured; PEN, Penicillium spiralis; PSE, Pseudomonas fluorescens; STA, Stachybotrys chartarum; STRE, Streptomyces coelicolor. Statistical significance difference compared to buffer control, p &lt; 0.05.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In human macrophages, only P. fluorescens (test for trend p = 0.000), S. caenioceras (p = 0.000), and positive control LPS stimulated a statistically significant IL-6 production, but the detected levels were much lower than those in mouse macrophages (Table 2). The maximum response was attained in 24 hr (Figure 2). Neither spores of B. eutropha nor any of the fungal strains increased the production of IL-6 in human macrophages at any dose (Table 2) or at any time point (Figure 2). Human epithelial cells produced moderately increased amounts of this cytokine after exposure to S. caenioceras and P. fluorescens (p-values for dose response, 0.025 and 0.000, respectively), whereas a weaker trend could be seen after exposure to A. versicolor (p = 0.005) and P. spiralis (p = 0.065; Table 3). Fungal strains were thus less potent than bacterial strains. In time-course study, only the spores of Sta. chartarum and P. fluorescens induced slight production of IL-6 in these cells (Figure 2). Interleukin-1β. Similar to the other measured cytokines, gram-negative bacterial LPS and P. fluorescens (test for trend p = 0.001) induced mouse RAW264.7 macrophages to produce equally high concentrations of IL-1β, and the trend for dose response was seen also for B. eutropha (p = 0.012) and S. caenioceras (p = 0.001; Table 1). The maximum was reached at 12 hr, and the levels declined thereafter (Figure 3). In addition, the spores of S. caenioceras induced slight IL-1β production with a similar time pattern. The spores of the fungal strains did not increase the production of IL-1β in mouse macrophages (Table 1, Figure 3). Both human cell lines failed to produce significant amounts of IL-1β at all (Table 2 and 3, Figure 3).

**Nitric oxide production.** LPS (not shown) and S. caenioceras induced increased NO production after 3 hr, and P. fluorescens after 6 hr of exposure in mouse RAW264.7 macrophages (Figure 4). The gram-negative bacterium P. fluorescens was again the most potent inducer among the microbes at low doses, whereas at the highest dose (100 ng/mL) the concentration of NO decreased (Table 1, Figure 4). The spores of gram-positive S. caenioceras elicited almost as high concentrations of NO in cell culture media as P. fluorescens, but with a clear increasing trend (p = 0.000). The other gram-positive bacteria and fungi did not cause any significant increase in NO production in this time frame. Inducible nitric oxide synthase was detectable by Western blotting in mouse cells, which produced increased amounts of NO (Figure 5). The expression was barely detectable at the highest concentrations (100 ng/mL) of P. fluorescens, and consequently with the low NO concentration in the culture medium (Table 1). This dose might
have caused cytotoxicity so rapidly that there was no time for induction of iNOS.

The spores of *Streptomyces* marginally increased the NO production in human macrophages in the time-course study. The concentrations were much lower than those induced in mouse macrophages. The increase was statistically significant at 6 and 24 hr (Fig. 4).

Human epithelial cells produced slightly increased amounts of NO only after the exposure to the spores of *Streptomyces* (not for trend *p* = 0.075). In the time-course study the difference compared to control cells was clearest at 24 hr (Table 3, Figure 4). Inducible nitric oxide synthase was not detectable by Western blotting from the human cell line (data not shown).

Cell viability. Bacteria decreased the viability of mouse macrophages in the same order as they induced the production of proinflammatory cytokines (Table 1). The gram-negative bacterium *P*. *fluorescens* was the most toxic to these cells, followed by the spores of gram-positive *Streptomyces* and *B. cereus*. With respect to the fungal spores, the cytotoxicity decreased in the order *Streptomyces* > *A. versicolor* > *P. spinulosa*, with the highest concentration (10⁷ spores/ml) for *Streptomyces*, 10⁶ spores/ml for *A. versicolor* and *P. spinulosa* clearly decreasing cell viability. The trend for dose response was statistically significant (*p*-values ranging from 0.000 to 0.005) in all but the case of *Streptomyces* (*p* = 0.166), for which the dose of 10⁷ spores/ml was not tested. The time-course of the cell viability by 10⁶ spores/ml showed that if the viability of the exposed cells was decreased, it took place in 3 hr (Figure 6). At 48 hr some recovery in cell numbers might even have taken place (Figure 6).

In human macrophages only the highest concentration (10⁷ microbes/ml) of all of the bacteria caused a 20–30% decrease in cell viability (Table 2). The cytotoxicity of the fungi decreased in the order *Streptomyces* > *A. versicolor* > *P. spinulosa*. The trend for dose response was statistically significant (*p*-value ranging from 0.000 to 0.019) in all but *P*. *fluorescens* (*p* = 0.207). Cell viability was not notably decreased at the concentration of 10⁶ microbes/ml. In all biochemical time-course analyses in human epithelial cells most microbes were clearly cytotoxic only at the concentration of 10⁷ microbes/ml. There was a statistically significant trend for dose response for all microbes (*p*-values ranging from 0.000 to 0.027), although *Streptomyces* was not cytotoxic in these cells (Table 3).

**Figure 2.** The time frame of IL-6 production after exposure to a single dose (10⁷ microbes/ml) of *A. versicolor* (ASPI), *P. spinulosa* (PEN), *Streptomyces* (STA), *B. cereus* (BAC), *S. californicus* (STR), and *P. fluorescens* (PSE) in *A*. mouse RAW264.7 macrophages, (B) human 2BSC cells, and (C) human A549 lung epithelial cells. Each column represents mean ± SEM of three independent experiments done in duplicate.

**Figure 3.** The time frame of IL-10 production after exposure to a single dose (10⁷ microbes/ml) of *A. versicolor* (ASPI), *P. spinulosa* (PEN), *Streptomyces* (STA), *B. cereus* (BAC), *S. californicus* (STR), and *P. fluorescens* (PSE) in *A*. mouse RAW264.7 macrophages, (B) human 2BSC cells, and (C) human A549 lung epithelial cells. Each column represents mean ± SEM of three independent experiments done in duplicate.

**Figure 4.** The time frame of nitrite production after exposure to a single dose (10⁷ microbes/ml) of *A. versicolor* (ASPI), *P. spinulosa* (PEN), *Streptomyces* (STA), *B. cereus* (BAC), *S. californicus* (STR), and *P. fluorescens* (PSE) in *A*. mouse RAW264.7 macrophages, (B) human 2BSC cells, and (C) human A549 lung epithelial cells. Each column represents mean ± SEM of three independent experiments done in duplicate.

**Discussion**

The current experiment reveals major differences between the inflammatory and cytotoxic potency of different groups of microbes present in indoor air. Interestingly, both gram-negative bacteria, *P*. *fluorescens*, and spores of gram-positive bacteria, *Streptomyces*, triggered profound effects in murine and human cells, whereas the spores of the
fungal used in this study lacked the capability to induce strong inflammatory responses. The time- and dose-dependent production of cytokine IL-6 in all three cell lines was induced only by the spores of gram-positive bacterium \textit{Streptomyces californicus}. Obviously, this microbe possesses an exceptional immunostimulatory capacity. This observation is in line with our earlier findings demonstrating immunological activity of both live and dead spores from different strains of streptomycetes in mouse macrophages (Hirvonen et al. 1997) and in human A549 cells (Jusila et al. 1999). This capacity, however, is not common to all gram-positive bacteria because \textit{B. cereus} did not activate either human or mouse cells to produce these mediators to the same extent as \textit{Streptomyces californicus}. The specific components or metabolites in the spores of streptomycetes causing these responses remain to be identified. For example, streptomycetes have complex lipid-sugar structures in their cell walls (Barrakov and Berghoff 1978), which bear a similarity to gram-negative bacterial LPSs. These bacteria also produce a vast variety of bioactive compounds as their secondary metabolites (Anderson and Wellington 2001). The spores of \textit{Streptomyces californicus} were also cytotoxic to mouse macrophages, but they decreased the viability of human cells only slightly.

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

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

In contrast to the bacterial strains, the spores of the fungal strains induced only a slight cytokine and NO production, and the cytokine profile was different. They were, however, cytotoxic, suggesting that the stimulation of cytokine production and cytotoxicity might not be associated. The cytotoxicity of fungi decreased in the order \textit{Sacccharomyces cerevisiae} > \textit{Aspergillus oryzae} > \textit{Penicillium sp.,} \textit{sp.}, but it did not differ notably from the cytotoxicity of bacteria. The predominant cytotoxicity of the spores of \textit{S. chartarum} over stimulation of the cytokine production in RAW264.7 macrophages has been observed previously (Ruotsalainen et al. 1998). Strain dependence of the toxicity of \textit{S. chartarum} is well known (Gravesen et al. 1994; Jarvis et al. 1998; Nikulin et al. 1997; Ruotsalainen et al. 1998), but the strain dependence seems to apply also for these other fungi. The cytotoxicity of the strain of \textit{S. chartarum}, \textit{A. versicolor}, and \textit{P. spinulosum} used in the present study varied when grown on different gyppsum boards (Murtomoziemi et al. 2001). The growth conditions of a microbe regulate its secondary metabolism, such as toxin production, and this regulation may be the key issue in understanding the microbial ecology.
of moisture-damaged building materials (Hirvonen et al. 2001). Altered growth and sporeulation environments for microbes may provoke cells to produce components and/or metabolites that trigger inflammatory responses and cytotoxicity. This implies that the potential to cause adverse effects may be site specific.

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

The present results demonstrate clearly that mouse macrophages reacted more than human macrophages to mold extracts from microfibers. The production of IL-1β and TNFα was induced in mouse macrophages but not in human macrophages and epithelial cells. In addition, the response was much more intense in mouse macrophages. All of these cells produced NO, but the NO synthase synthesis was induced only in mouse macrophages, even by LPS. Among the measured inflammatory mediators, the production of the proinflammatory cytokine IL-6 was the most consistent indicator of inflammatory reactions. Its production was induced in all cell lines. The apparent insensitivity of human cells is most likely attributable to species differences in the regulation of NO and cytokine production. Species differences have been described in activation of lung macrophages to produce NO (Dörger et al. 1997; Josch et al. 1997), and the regulation of the expression of iNOS is different in mice and human cells (DeVera et al. 1996; Ganster et al. 2001; Löwenstein et al. 1995; Xie et al. 1993). Moreover, the maximal cytokine production requires certain coincidental stimuli (Xie et al. 1993; Löwenstein et al. 1993; Gutierrez et al. 1995), and in vitro conditions cannot mimic such extracellular milieu as these found in the lungs. Although the human cell lines were printed with interleukin-1, this alone might not be sufficient; other priming agents would also be needed. Interleukin-1 clearly potentiated the response of Staph. aureus in A549 lung epithelial cell line in our previous study (Jusila et al. 1999). Human cells were also more resistant to the microbial-induced cytotoxicity than mouse macrophages. However, the setup of the experiments did not allow for the evaluation of the cellular mechanisms of these effects. The microbe-induced production of IL-6 in the human cell lines seems to be independent of the production of IL-1β and TNFα, although this needs to be confirmed by more sensitive methods.

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

References

Lettmann U, Ailey DD, Renn P, Snowin AG, Snyder DH,
Articles | Hutunen et al.


Metabolite profiles of *Stachybotrys* isolates from water-damaged buildings and their induction of inflammatory mediators and cytotoxicity in macrophages

Kristian Fog Nielsen¹,², Kati Huttunen³, Anne Hyyräinen³, Birgitte Andersen¹, Bruce B. Jarvis⁴ & Maija-Riutta Hirvonen³

¹The Mycology Group, BioCentrum-DTU, Building 221, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark; ²Energy and Indoor Climate Division, Danish Building Research Institute, Dr. Neergaardsvej 15, DK-2970 Horsholm, Denmark; ³Department of Environmental Health, National Public Health Institute, P.O. Box 95, FIN-70701 Kuopio, Finland; ⁴Department of Chemistry and Biochemistry and the Joint Institute for Food Safety and Applied Nutrition (JIFSAN), University of Maryland, College Park, MD 20742, USA

Received 19 February; accepted in revised form 15 March 2002

Abstract

The metabolite profiles of 20 *Stachybotrys* spp. isolates from Finnish water-damaged buildings were compared with their biological activities. Effects of purified compounds on cytotoxicity and production of inflammatory mediators such as nitric oxide, IL-6 and TNFα in murine RAW264.7 macrophage cells were studied. The 11 isolates belonging to the satratoxin-producing chemotype were highly cytotoxic to the macrophages. The isolates inducing inflammatory mediators all belonged to the atranone-producing chemotype, but pure atranones B and D did not elicit a response in the bioassay. Altogether, cytotoxicity of *Stachybotrys* sp. isolates appear to be related to satratoxin production whereas the specific component inducing inflammatory responses in atranone-producing isolates remains obscure.

Keywords: Atranones, satratoxins, mycotoxin, *Stachybotrys*, inflammation, TNFα, IL-6.

Introduction

Growth of *Stachybotrys chartarum* (Ehrenb. ex Link) Hughes (= *S. atra* Corda) [1] in water-damaged buildings is associated with adverse health effects, including idiopathic pulmonary hemosiderosis (IPH) in infants [2–5].

It was recently shown that there are significant differences in the biological activity of *Stachybotrys* isolates from Finnish water-damaged buildings [6]. The isolates could be divided into two groups: one that was highly cytotoxic to the mouse RAW264.7 macrophages and the other, less cytotoxic but one that induced a strong inflammatory response i.e. the production of nitric oxide (NO), tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6) in these cells [6–9].

Between 30 and 40% of the *S. chartarum* isolates from water-damaged buildings produce macrocyclic trichotheccenes [4, 10]. The macrocyclic trichotheccenes are biosynthetically derived from the simple trichotheccene, trichodermol. Trichodermol can be acetylated into trichoderrmin and/or oxidized further to verrucarol, a simple trichotheccene that is elaborated further to the trichoverroids that are the direct precursors to the class of macrocyclic trichotheccenes, e.g., roridins, the verrucarins, and satratoxins [11, 12]. The macrocyclic forms are typically at least 10–100 times as cytotoxic as the simple ones, suppress the immune system, are highly cytotoxic (LD₅₀ in mice ~1 mg/kg) and are believed to be responsible for stachybotryotoxicosis in animals [4].

Published in 2002.
The remaining 60–70% of S. chartarum isolates seem to stop trichothecene biosynthesis at trichodermin/trichodermin stage [10] and consistently produce a series of new diterpenoids named atranones and their dolaslanee precursors [10, 13]. No macrocyclic trichothecene-producing isolate of S. chartarum has ever been found to produce atranones or dolaslanes [13]. The atranone-producing isolates can be subdivided further into two groups, with 70–80% belonging to the classically described S. chartarum [1] and 20–30% belonging to an apparent new species that is morphologically close to S. chartarum, that we refer to as Stachybotrys sp. type A [10].

All S. chartarum isolates produce a series of spirocyclic dirimanes (often >20 congeners) that are produced at considerably higher levels than those of either the atranones or trichothecenes. The spirocyclic dirimanes exhibit a wide variety of biological properties, *inter alia* complement inhibition, anti-thrombotic activity, fibrinolytical activity, neurotoxic effects, and Tumor-Necrosis-Factor liberation inhibitor [14].

In the current study, we aimed to investigate whether there was a relationship between metabolite profiles and the biological activities of the two different chemotype groups previously investigated by Ruotsalainen et al. [6]. Cytotoxicity and the ability of the isolates to induce the production of NO and cytokines (TNFα and/or IL-6) from several pure components in the RAW264.7 macrophage cell were also studied.

### Experimental

#### Microbiology and chemical analyses

Twenty of the 21 *Stachybotrys* isolates from the original study [6] were received on 2% malt extract agar (MEA) from National Public Health Institute, Kuopio, Finland. Each isolate was inoculated as three-point-inoculation onto potato sucrose agar, PSA (used for metabolite profiling) and cornmeal agar, CMA (for micro morphology). They were incubated at 25 °C for 7 days in the dark, and identified according to Jong and Davis [1] and Andersen et al. [10]. All isolates used in this study are held at the culture collection at Biocentrum-DTU, Technical University of Denmark.

*Stachybotrys* cultures were extracted and analyzed by HPLC after polyethyleneimine silica cleanup [10] and then by GC-MS/MS after hydrolysis and heptafluorobutyl derivatization [10, 15].

**Cytotoxicity and inflammatory responses to pure Stachybotrys compounds**

The RAW 264.7 macrophages were cultivated as described by Ruotsalainen et al. [6]. Briefly, the cells were cultured in 2-ml wells, allowed to adhere and stabilize for 24 hours and exposed to different doses of pure mycotoxins (0.003 ng/ml to 56 μg/ml). Sample preparation: iso-satratoxin F, verrucarin J, atranone B and atranone D (available from previous studies in the laboratory of BBJ) were dissolved in methanol for 1 hr, diluted (1:10) in HBSS buffer, and 100 μl of this solution was added to each well. After 24 hours the exposure was stopped, and the proportion of living macrophages was assessed according to MTT-method. The production of NO and cytokines IL-6 and TNFα were measured from the culture medium as described by Ruotsalainen et al. [6].

#### Results and discussion

The chemical analyses showed that 11 of the 20 *Stachybotrys* isolates produced satratoxins and the remaining isolates produced atranones and dolaslanes with 6-hydroxydolaslanee-3E,8E,12-trien-14-one being the marker compound for this class of metabolites [13] (Table 1). All the isolates that were capable of producing satratoxins were previously shown to kill between 55 and 85% of the macrophages at the concentration 10³ spores/ml [6].

All of the producers of 6-hydroxydolaslanee-3E,8E,12-trien-14-one, except HT523, were non-cytotoxic to the macrophages compared to the control cells at the dose of 10³ spores/ml in the earlier study [6]. Instead these isolates induced production of ROS, NO, IL-6 and TNFα at the dose of 10⁹ spores/ml [6]. HT523 apparently induces a response similar to that of the satratoxigenic strains, but has a metabolite profile similar to that of the non-satratoxigenic strains; isolate HT523 may produce a cytotoxic compound(s) as yet uncharacterized.

Two other isolates exhibited anomalies. The non-satratoxigenic isolate HT520, upon hydrolys of the culture, produced a small quantity of verrucarol, and the non-satratoxigenic isolate HT16 exhibited no significant activity in the TNFα and IL-6 screens. Interestingly, this latter isolate is the only member of the group A non-satratoxigenic isolates in this study.

As shown in a number of studies, the macrocyclic trichothecones (satratoxins, roridin E, and verrucarin...
Table 1. Results from the metabolite profiling of Stachybotrys isolates on PSA agar compared with results of biologically activity from the previous study.

<table>
<thead>
<tr>
<th>KTL no.</th>
<th>BTT no.</th>
<th>Satratoxins G and H</th>
<th>Arranones</th>
<th>Trichodermol</th>
<th>Verrucarol</th>
<th>Induction of TNFα and IL-6</th>
<th>Cytotoxicity (% dead cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT530</td>
<td>9713</td>
<td>Yes</td>
<td>ND</td>
<td>****</td>
<td>**</td>
<td>NS</td>
<td>85</td>
</tr>
<tr>
<td>HT72</td>
<td>9703</td>
<td>Yes</td>
<td>ND</td>
<td>*</td>
<td>*</td>
<td>NS</td>
<td>85</td>
</tr>
<tr>
<td>HT248</td>
<td>9698</td>
<td>Yes</td>
<td>ND</td>
<td>**</td>
<td>NS</td>
<td>NS</td>
<td>85</td>
</tr>
<tr>
<td>HT522</td>
<td>9710</td>
<td>Yes</td>
<td>ND</td>
<td>****</td>
<td>NS</td>
<td>NS</td>
<td>85</td>
</tr>
<tr>
<td>HT296</td>
<td>9697</td>
<td>Yes</td>
<td>ND</td>
<td>ND</td>
<td>***</td>
<td>NS</td>
<td>80</td>
</tr>
<tr>
<td>HT386</td>
<td>9704</td>
<td>Yes</td>
<td>ND</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td>80</td>
</tr>
<tr>
<td>HT387</td>
<td>9690</td>
<td>Yes</td>
<td>ND</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td>80</td>
</tr>
<tr>
<td>HT569</td>
<td>9702</td>
<td>Yes</td>
<td>ND</td>
<td>**</td>
<td>****</td>
<td>NS</td>
<td>80</td>
</tr>
<tr>
<td>HT391</td>
<td>9709</td>
<td>Yes</td>
<td>ND</td>
<td>*</td>
<td>***</td>
<td>NS</td>
<td>80</td>
</tr>
<tr>
<td>HT471</td>
<td>9700</td>
<td>Yes</td>
<td>ND</td>
<td>*</td>
<td>***</td>
<td>NS</td>
<td>75</td>
</tr>
<tr>
<td>HT165</td>
<td>9696</td>
<td>Yes</td>
<td>ND</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td>55</td>
</tr>
<tr>
<td>HT523</td>
<td>9708</td>
<td>ND</td>
<td>yes</td>
<td>ND</td>
<td>ND</td>
<td>NS</td>
<td>85</td>
</tr>
<tr>
<td>HT166</td>
<td>9714</td>
<td>ND</td>
<td>yes</td>
<td>ND</td>
<td>ND</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HT435</td>
<td>9691</td>
<td>ND</td>
<td>yes</td>
<td>ND</td>
<td>ND</td>
<td>S</td>
<td>NS</td>
</tr>
<tr>
<td>HT502</td>
<td>9707</td>
<td>ND</td>
<td>yes</td>
<td>ND</td>
<td>ND</td>
<td>S</td>
<td>NS</td>
</tr>
<tr>
<td>HT503</td>
<td>9705</td>
<td>ND</td>
<td>yes</td>
<td>ND</td>
<td>ND</td>
<td>S</td>
<td>NS</td>
</tr>
<tr>
<td>HT518</td>
<td>9695</td>
<td>ND</td>
<td>yes</td>
<td>ND</td>
<td>ND</td>
<td>S</td>
<td>NS</td>
</tr>
<tr>
<td>HT513</td>
<td>9694</td>
<td>ND</td>
<td>yes</td>
<td>ND</td>
<td>ND</td>
<td>S</td>
<td>NS</td>
</tr>
<tr>
<td>HT520</td>
<td>9701</td>
<td>ND</td>
<td>yes</td>
<td>***</td>
<td>*</td>
<td>S</td>
<td>NS</td>
</tr>
<tr>
<td>HT401</td>
<td>9693</td>
<td>ND</td>
<td>yes</td>
<td>*</td>
<td>ND</td>
<td>S</td>
<td>NS</td>
</tr>
</tbody>
</table>

1 6-hydroxyo-lapella 3E,8E, 12-tien-14-one.
2 Determined by HPLC-DAD.
3 Determined by GC-MS/MS.
4 At 10⁴ spores/ml. From Ruusulainen et al. [6].
5 At 10⁵ spores/ml. From Ruusulainen et al. [6].
6 Isolate Stachybotrys sp. Type A [10].
7 <10 μg/cm², 20-30 μg/cm², 30-50 μg/cm², 50-100 μg/cm², from PSA culture.
ND: not detected. NS: not significantly different from control. S: significantly higher than control.

J) from the satratoxigenic strains are highly cytotoxic to macrophages; whereas, arranones B and D (from the arranone-producers) are only slightly cytotoxic compared to solvent blanks (Figure 1). This indicates very clearly that the cytotoxicity of these S. chartarum isolates is related to the ability of the fungi to produce macrocyclic trichotheccenes, principally satratoxins G and H. None of the pure compounds i.e., iso-satratoxin F, and verrucarol J or arranones B and D, induced either NO or cytokine production (cf. solvent blanks) in the macrophages up to doses as high as 50 μg/ml. Thus, the specific agent triggering the detected strong inflammatory response is still unidentified. However, the wide variety the spiriocyclic drimanes, that are known to exhibit several biological properties is a feasible suggestion. Identification of these components is of special interest since high induction (up to 1 ng/ml [6]) of e.g. TNFα is known to be a significant factor for the induction of lung damage [16]. Nikulin et al. [17, 18] suggested that the satratoxins were the main reason of the lung tissue inflammation when they exposed mice with S. chartarum spores from a satratoxin producer (#72) and a non-satratoxin producer (#29, produces arranones [10]). But the latter isolate also produced significantly smaller quantities of the spiriocyclic drimanes, making these components an alternative cause. It should also be noted that this inflammatory agent(s) may be present in the satratoxin-producing isolates, but the potent cytotoxins produced by these isolates kill the macrophages before they can induce an inflammatory response. The significant lack of both cytotoxic and inflammatory responses from isolate HT16, suggests that these responses are not due to the particle effect since the spores are nearly identical in size and shape to S. chartarum, although it might be due to
compounds inhibiting TNFα liberation [19]. This interpretation is also supported by earlier observations by Mason et al. [20] demonstrating very different effects between the pure toxin, iso-satratoxin F and S. chartarum spores on lung homeostasis and surfactant production.

Altogether, our results show very clearly that the Stachybotrys spp. isolates can be divided into two different groups: highly cytotoxic ones that produce macrocyclic trichotheccenes, and another group producing atranones, (and sometimes simple trichotheccenes, e.g., trichodermin and trichodermin) associated with induction of strong inflammatory responses in macrophages. However, the present study demonstrates that the production of inflammatory mediators in macrophages by the spores of atranone producing isolates is not triggered by pure atranones. Thus, further studies on different strains are needed to identify the possible components causing the inflammatory responses in immunological cells.

Acknowledgments

The authors wish to thank Ms. Heli Martikainen for excellent technical assistance. The study was supported by the Danish research program Mould in Buildings and the Finnish Research Programme on Environmental Health SYTTY.

BBJ wishes to thank the Technical University of Denmark and the Danish National Bank for their kind support during the sabbatical year 1999–2000.

References

6. Ruotsalainen M, Hirvonen M-R, Nevalainen A, Meklin T, Savolainen K. Cytotoxicity, production of reactive oxygen species and cytokines induced by different strains of Stachy-
   necrosis factor, and interleukin-6 in RAW 264.7 macrophages by streptomyces from indoor air of mouldy houses. Arch
8. Hirvonen M-R, Ruotsalainen M, Savolainen K, Nevalainen A. Effect of viability of actinomycete spores on their ability
   to stimulate production of nitric oxide and reactive oxygen species in RAW 264.7 macrophages. Toxicology 1997;
   160: 1493.
10. Andersen B, Nielsen KF, Jarvis BB. Characterisation of Stachybotrys from water-damaged buildings based on morpho-
11. Desjardins AE, Hohn T, McCormick SP. Trichothecene Biosynthesis in Fusarium Species: chemistry, genetics, and
12. Fekete C, Logrieco A, Giczy G, Homok L. Screening of fungi for the presence of the trichodine synthase encoding
13. Hinkley SF, Mazuela EP, Pettinger JC, Lam Y-F, Jarvis BB. Atranones A-G, from the toxigenic mold Stachybotrys char-
15. Nielsen KF, Thrane U. A fast method for detection of tri-
   chothecenes in fungal cultures using gas chromatography-
   tribution of tumor necrosis factor-alpha to pulmonary cytokine
   expression and lung injury after hemorrhage and resuscitation.
17. Nikulin M, Reijula K, Jarvis BB, Veijalainen P, Hintikka E-
    L. Effects of intranasal exposure to spores of Stachybotrys atra
19. Tomishige T, Miyazaki K. Tumor necrosis factor liberation inhi-
    bitors containing sesquiterpene analogs or their salts. 1992.
20. Mason CD, Rand TG, Oulton M, MacDonald JM, Scott JE.
    Effects of Stachybotrys chartarum (atra) conidia and isolated
    toxin on lung surfactant production and homeostasis. Natural

Address for correspondence: Kristian Fog Nielsen, The Mycology Group, BioCentrum-DTU, Building 221, Technical University of
Denmark, DK-2800 Kgs. Lyngby, Denmark
Phone: +45 4525 26 02; Fax: +45 4588 49 22
E-mail: kristian.f.nielsen@biocentrum.dtu.dk
Synergistic interaction in simultaneous exposure to *Streptomyces californicus* and
*Stachybotrys chartarum*. Submitted.
Synergistic Interaction in Simultaneous Exposure to *Streptomyces californicus* and *Stachybotrys chartarum*

Kati Huttunen, a,b Jukka Pelkonen b, Kristian Fogg Nielsen c, Ulla Nuutinen b, Juha Jussila b, and Maija-Riitta Hirvonen a

a Division of Environmental Health, National Public Health Institute, Kuopio, Finland

b University of Kuopio, Kuopio, Finland

c Biocentrum-DTU, Technical University of Denmark, Lyngby, Denmark

Corresponding author:

Kati Huttunen

Division of Environmental Health

National Public Health Institute

P.O. Box 95, FIN-70701 Kuopio, Finland

Telephone: +358 17 201320 / Telefax: +358 17 201265

Kati.Huttunen@ktl.fi
Abstract

The microbial exposure associated with health complaints in moldy houses consists of a heterogeneous group of components, including both living and dead bacteria, fungi, their metabolites and active compounds. However, little is known about the interactions between different microbes and their metabolites, although the cytotoxicity and inflammatory potential of certain microbes has been reported. In this study we investigated the inflammatory responses of mouse RAW264.7 macrophages after exposure to six indoor air microbes (Aspergillus versicolor, Penicillium spinulosum, Stachybotrys chartarum, Bacillus cereus, Mycobacterium terrae and Pseudomonas fluorescens) alone and together with actinomycete Streptomyces californicus. The production of nitric oxide, levels of pro-inflammatory cytokines TNF-α and IL-6, and cytotoxicity were measured. The co-exposure to Stachybotrys chartarum and Streptomyces californicus caused a synergistic increase in the production of IL-6. In further experiments, the metabolites from S. chartarum or closely related fungi (atranones B and E, satratoxin G, trichodermin, 7-α-hydroxytrichodermol, staplabin and SMTP-7) as well as the known fungal toxins (sterigmatocystin, citrinin and ochratoxin A) were all tested together with Str. californicus. The testing revealed a similar synergistic response in cytotoxicity, TNF-α and IL-6 production after co-exposure to Str. californicus with both trichodermin and 7-α-hydroxytrichodermol. Finally, the synergistic inflammatory response caused by Str. californicus and trichodermin together was studied by analyzing for the presence of NF-κB in nuclear extracts of the exposed cells. The exposure to Str. californicus induced the binding of NF-κB proteins to the NF-κB consensus sequence as well as to the natural NF-κB site of the IL-6 promoter. Adding trichodermin to the exposure did not increase the DNA binding.

Keywords: Interaction; Bacteria; Fungi; Mycotoxins; Inflammation; Toxicity
INTRODUCTION

Microbial exposure in moldy houses consists of a heterogeneous group of compounds, including both living and dead bacteria, fungi, their metabolites and active components as well as fungal micro-particles (0.3-1 μm in diameter) (Gorny et al. 2002, Kildesø et al. 2000). Interactions between the different exposures in a moisture damaged house are inevitable, knowing that alone the spores of a single fungal species may contain various metabolites, and the moisture damaged site is always a habitat of more than one microbial species (Andersson et al. 1997, Hyvärinen et al. 2002, Nielsen et al. 1999). Since many of the secondary metabolites are thought to be involved in chemical signaling between organisms or species, the production of some of these active metabolites may be enhanced or inhibited when the microbes interact (Christophersen, 1996). However, little is known about the interactions between different elements of microbial exposure although the inflammatory potential of some microbes and microbial components is acknowledged (Fogelmark et al. 2001, Jagieło et al. 1996).

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

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

Our earlier studies comparing the effects of fungal and bacterial exposure in vitro indicated that the importance of exposure to bacteria, especially gram positive actinomycetes, might have been underestimated (Huttunen et al. 2003). Since the numbers of bacteria in indoor air are frequently higher than fungi, the bacterial exposure and its interaction with the fungi is therefore of great importance (Flannigan et al. 1991). Actinomycetes have sparked interest in many aspects of the moldy house problem. Their occurrence is considered to be indicative of moisture damage, several studies have found actinomycetes consistently from moldy house samples (Hyvärinen et al. 2002, Nevalainen et al. 1991), and very active metabolites have been isolated from actinomycetes rich samples (Andersson et al. 1998). Furthermore, the inflammatory potential of this gram positive sporulating bacteria has been studied in cell culture studies and in a mouse model, both studies revealing its high potential for inducing inflammatory effects (Huttunen et al. 2003, Jussila et al. 1999, Jussila et al. 2001). The frequent occurrence of actinomycetes in moisture damaged materials means that it
often shares its habitat with other microbial species, especially fungal genera like *Aspergillus*, *Penicillium* and *Stachybotrys* (Hyvärinen et al. 2002).

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

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

Microbial strains. The spores of three fungal strains, *Aspergillus versicolor* (HT486), *Penicillium spinulosum* (HT581) and *Stachybotrys chartarum* (HT580= IBT 9706), and four bacterial strains, *Mycobacterium terrae* (BA26), *Bacillus cereus* (B64), *Pseudomonas fluorescens* (N78) and *Streptomyces californicus* (A4), were all isolated and identified in previous studies (Huttunen et al. 2001, 2003). The fungal strains were cultured on 2% malt extract agar (MEA) and bacterial strains on tryptone yeast glucose agar (TYG) as a dense culture and incubated in the dark at 25°C for 7 days and 20°C for 5 days, respectively. All strains are available from the culture collection of the National Public Health Institute, Kuopio, Finland.

Fungal metabolites. Sterigmatocystin, citrinin, and ochratoxin A were obtained from Sigma (St. Louis, MO). Trichodermin was a gift from Løvens Kemiske Fabrik A/S (Ballerup, Denmark); Atrazones B and E, 7-α-hydroxytrichodermol, and satratoxin G were a gift from Professor Bruce B. Jarvis (University of Maryland, MD); stapablin and SMTP-7 were a gift from Professor Keiji Hasumi (Tokyo Noko University, Japan). Fungal metabolites were dissolved in methanol and diluted further in HBSS, the final concentration of methanol being less than 1%.

The samples from the microbial/fungal cultures (10⁹ spores/ml) were extracted by ethyl acetate (analytical grade) evaporated to dryness, redissolved in methanol and filtered through a 0.45 μl PTFE syringe filter (Titan 44513-PL, SRI, Eatontown, NJ). Subsequently it was analyzed by reversed phase chromatography on an Agilent 1100 liquid chromatographic (LC) (Waldbonn, Germany) system with a diode array detector (DAD) coupled to a Micromass LCT (Manchester, UK) orthogonal time of flight mass spectrometer operated in positive electrospray mode scanning from m/z 100 to 2000. Peaks in the UV and MS traces
were compared with reference standards 550 fungal metabolite reference standard from the Mycology Group metabolite database (Nielsen and Smedsgaard, in press).

**Cell culture.** The mouse macrophage cell line (RAW264.7) was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured at 37°C in 5% CO₂ atmosphere in RPMI 1640 –medium supplemented with 10% of heat inactivated fetal bovine serum (FBS), 1% of L-glutamine and 1% of penicillin-streptomycin (all from Gibco BRL, Paisley, UK). The macrophages (5 x 10⁵ cells/ml) were dispensed to 6-well plates, 2 ml/well. The cells were allowed to adhere for 24 hours, and fresh complete medium was added before exposure. The cells were exposed to the combination of *Str. californicus* and graded doses of either microbes or fungal metabolites in 200 μl of HBSS. After the exposure, the adherent cells were resuspended in the culture medium by scraping, the viability of the cells was assessed and the cell suspension was centrifuged (5 min, 8000 rpm) in order to separate the cells from the culture medium. The samples were stored at -80°C for the subsequent analyses.

**Study design.** The macrophages were exposed to three doses (10⁴, 10⁵ and 10⁶ spores or bacterial cells/ml) of each microbe alone and in combination with *Str. californicus* at a dose of 10⁵ spores/ml for 24 hours. The interaction with the isolated fungal metabolites was tested by exposing the cells to three doses (10, 100 and 1000 ng/ml) of each metabolite, except for satratoxin G which was tested with doses 1, 10 and 100 ng/ml due to its toxicity. Trichodermin and its analogue, 7-α-hydroxytrichodermin were tested further also with additional doses (5, 50 and 500 ng/ml) in order to better define the dose-response. The control cells were exposed to carrier buffer, 10% methanol in HBSS. The nuclear extracts for EMSA were prepared from cells exposed to two doses (100 and 500 ng/ml) of trichodermin and 10⁵ spores/ml *Str. californicus* for 24 hours both alone and together.
**Nitrite analysis.** Nitric oxide was measured spectrophotometrically as the stable metabolite, nitrite (NO₂) according to the Griess method (Green et al. 1982). Briefly, Griess reagent (1 % sulphathiazole and 0.1 % naphthylethylenediamine dihydrochloride in 2 % phosphoric acid) was mixed 1:1 with samples of the cell culture medium. Nitrite forms a colored chromophore with the reagent, with an absorbance maximum at wavelength of 543 nm, which was measured with an ELISA microplate reader (iEMS Reader MF, Labsystems, Turku, Finland). The production of nitrite was quantified by comparing the results with absorbances of standard solutions of sodium nitrite. Samples of at least three independent experiments were analyzed in duplicate.

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

**Cell viability.** The viability of the macrophages was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) –test to detect living mitochondria (Mosmann, 1983). Live mitochondria can transform MTT (Sigma Chemical Co, MO, USA) to formazan, which can be measured with a spectrophotometer. The proportion of viable cells in exposed samples was compared to control samples, where the viability was measured by staining the cells with Trypan Blue solution. Samples of at least three independent experiments were analyzed in duplicate.

**Preparation of nuclear extracts.** For the preparation of nuclear extracts, the exposed cells were washed with PBS and suspended in a hypotonic buffer (1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 10 mM HEPES). After a 5 min incubation on ice, 0.08% NP-40 was added and the
suspension was incubated for a further 2 min before centrifugation (400 g, 2 min, 4°C). Pellets containing the nuclei were suspended in hypertonic buffer (25% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 420 mM NaCl, 20 mM HEPES) and incubated at 4°C for 30 min with gentle shaking. Finally, samples were centrifuged (25000 g, 20 min, 4°C) and supernatants were stored at -80°C. The amount of nuclear proteins was analyzed with Lowry’s method (DC protein assay, BioRad Laboratories).

**EMSA assay.** Double-stranded oligonucleotide containing the NF-κB consensus binding site was 5'-AGT TGA GGG GAC TTT CCC AGG C-3' (Promega, Madison, WI, USA).

Single-stranded oligonucleotides containing the natural NF-κB binding motif of the mouse IL-6 promoter (sense 5'-AAA TGT GGG ATT TTC CCA TGA GTC-3', anti-sense 5'-GAC TCA TGG GAA AAT CCC ACA TTT-3') were annealed before labeling.

The 173 bp region (-43 to -216) of the mouse IL-6 promoter was amplified by half-nested PCR using BALB/c mouse genomic DNA as the template. The primers were as follows:

IL-6 sense  
5'-CGACGTCACATTGTGCAATC-3'

IL-6 anti-sense1  
5'-CAGAATGAGCTACAGACATC-3'

IL-6 anti-sense2  
5'-GTGGGAGTGGTATCCTCTG-3'

The PCR product was extracted from low-melting agarose by the phenol-chloroform method. Double-stranded DNA oligonucleotides and the PCR product were labeled with [γ-³²P]ATP (3000 Ci/mmol; Amersham Pharmacia Biotech, Roosendaal, Netherlands) using T4-polynucleotide kinase (MBI Fermentas, Hanover, MD, USA). Labeled probes were separated using Probe Quant™G-50 micro columns (Amersham Pharmacia Biotech, Roosendaal, Netherlands) prior to their use in EMSA experiments.

Extracted nuclear proteins (6 μg protein per reaction) were incubated for 20 minutes with [γ-³²P]ATP labeled probes in binding buffer (10% glycerol, 1 mM DTT, 1 mM EDTA, 25 mM HEPES, 100 mM NaCl) and 1.5 μg poly(dI-dC) in a final reaction volume of 20 μl. The
protein-DNA complexes were separated (25 mA, 90-120 min) on a high ionic strength gel (6 % acrylamide) in running buffer (50 mM Tris, 380 mM glycine, 1 mM EDTA). After electrophoresis, the gel was dried (1h, 60°C) and exposed to autoradiography film for 1-2 days at -80°C.

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

Statistical analysis. The data were statistically analyzed using analysis of variance (ANOVA) (SPSS, version 7.51, SPSS Inc.) and Tukey’s test. The difference was considered to be statistically significant at P<0.05.

RESULTS

Simultaneous exposure to Str. californicus and selected microbes. A low dose of Str. californicus (10⁵ spores/ml) alone and in combination with three different doses of A. versicolor, P. spinulosum, S. chartarum spores, and bacterial strains, B. cereus, M. terrae and P. fluorescens were studied to find out if the microbes were interacting during the exposure.

Cytokine production. Exposure to Str. californicus at the dose of 10⁵ spores/ml alone caused a strong TNF-α production and a moderate IL-6 production. Out of the six tested microbes, only P. fluorescens caused a markedly increased, dose-dependent IL-6 production (p = 0.000) without co-exposure, whereas TNF-α production was induced by all the microbes, B. cereus and P. fluorescens being the most potent (p=0.067 and p=0.000, respectively). Interestingly, simultaneous exposure to the low dose of Str. californicus (10⁵ spores/ml) together with S.
charitarum caused the IL-6 to be elevated dose-dependently up to 150 ± 170 pg/ml (p=0.086). This same effect was not seen with the other measured cytokine, TNF-α. The co-exposure to the other tested microbes did not potentiate any of the measured cytokine responses to spores of Str. californicus (Table 1).

Nitric oxide production. The dose of 10⁵ spores/ml of Str. californicus on their own caused only minor production of nitric oxide (NO). Out of the single exposures, only P. fluorescens caused an intense production of NO (p=0.000). None of the tested combinations caused a significant increase in NO production, but there was a slight addition of response with simultaneous exposure to P. spinulosum (p=0.022) together with Str. californicus spores (Table 1).

Cell viability. Single exposure to the spores of Str. californicus at the dose of 10⁵ spores/ml caused only a moderate decrease in cell viability. Out of the tested microbes, S. chartarum (p=0.000) and P. fluorescens (p=0.000) were the most cytotoxic, followed by A. versicolor (p=0.000), B. cereus (p=0.001), P. spinulosum (p=0.000) and M. terrae. A synergistic interaction was detected in the cytotoxicity to the cells in simultaneous exposure to S. chartarum (p=0.009), A. versicolor (p=0.008) P. spinulosum (0.023) or P. fluorescens (p=0.023) together with Str. californicus spores (Table 1).

Detected metabolites. The A. versicolor extract was dominated by sterigmatocystin, 5-methoxy sterigmatocystin, versicolorins and other members of the sterigmatocystin biosynthetic pathway. The P. spinulosum extract contained very few and low quantities of metabolites, out of which only 6-methyl-citreoisocumarin could be identified. Two unknown metabolites with MW of 265 and 348 were also detected, which suggested that this strain is rather a Penicillium glabrum than a P. spinulosum. The S. chartarum extract was dominated by 7 spirocyclic dimeranes. The atranones 3,4-epoxy-6-hydroxy-dolabella-7,12-diene-one; 6-hydroxy-dolaballa-3,7,12-trien-14-one; and atranone A were also present, whereas no
trichotheccenes (trichodermin, satratoxins etc.) were detected. In addition, two unknown metabolites with presumed MW of 531 Da were also detected. In the *P. fluorescens* extract, a cyclic peptide with a MW of 1126 was detected along with 3 minor analogues with MW 1111, 1153 and 1151 Da. Nothing was detected in the *B. cereus*, *M. terrae* and the *Str. californicus* extracts.

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

**Cytokine production.** The tested metabolites of *S. chartarum* caused significantly different cytokine responses in RAW264.7 cells. Atranones B and E caused hardly any production of TNF-α or IL-6 in macrophages by any tested doses whereas trichodermin induced a dose-dependent, significant increase in TNF-α production (*p* = 0.000) and slight but significant IL-6 production (*p* = 0.000) alone and this response was markedly increased together with spores of *Str. californicus* (*p* = 0.000 for both TNF-α and IL-6) (Fig. 1). The closely related component, 7-α-hydroxytrichodermol triggered similar effects on IL-6 and TNF-α production (*p* = 0.000 for both), though the dose response curve differed from that obtained with trichodermin. The cytokine production induced by trichodermin tended to decline slightly with the highest doses, possibly due to the cytotoxicity of the exposure, whereas the exposure to 7-α-hydroxytrichodermol increased the levels of produced cytokines dose-dependently at all tested doses (Fig. 1). Satratoxin G was sufficiently toxic to significantly decrease the production of TNF-α (*p* = 0.000) when compared to control cells. Staplabin, SMTP-7,
sterigmatocystin, citrinin and ochratoxin did not induce any significant production of IL-6 in RAW264.7 macrophages and only ochratoxin A induced a slight increase in TNF-α production. None of these mycotoxins added to the inflammatory effect of the spores of \textit{Str. californicus}, in contrast even a slight decreasing trend was detected in TNF-α production.

**Nitric oxide production.** Exposure to low dose of \textit{Str. californicus} (10^5 spores /ml) elevated slightly, but non-significantly, the levels of nitrite in the culture medium of the macrophages. None of the tested microbial metabolites induced any significant production of nitric oxide alone, and only atranone E induced a slightly increased NO production together with the spores of \textit{Str. californicus}. Interestingly, cells exposed to trichodermin alone produced somewhat higher amounts of NO than cells exposed to both trichodermin and the actinomycete.

**Cell viability.** The cytotoxicity of the metabolites of \textit{S. chartarum} was paralleled with the potency to induce inflammatory effects. Atranones were not cytotoxic either alone or when combined with spores of \textit{Str. californicus}. The doses over 50 ng/ml of trichodermin were significantly cytotoxic (p = 0.000) and also amplified the cytotoxic effects of \textit{Str. californicus} spores (p = 0.051), whereas for the 7-α-hydroxytrichodermin, the doses over 500 ng/ml were significantly cytotoxic (p = 0.000) to exposed cells but no amplifying effect could be detected in co-exposure with the actinomycete (Fig. 2). Satratoxin G was highly cytotoxic to macrophages (p = 0.000), and the toxicity was somewhat increased with \textit{Str. californicus} (p = 0.051). The cytotoxic effect of satratoxin G could be seen already at ten times lower doses than the other tested toxins. Exposure to staplin (p = 0.000) and SMTP-7 (p = 0.001) decreased slightly, but significantly the viability of the macrophages, whereas ochratoxin, citrinin and sterigmatocystin did not cause excessive cell death alone or when combined with the spores of \textit{Str. californicus}. (Fig. 2).
Role of NF-κB in the microbial exposure and in the regulation of IL-6 production.

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

Binding of nuclear proteins to cκB oligonucleotide. Str. californicus exposure caused a major increase in DNA binding of all three nuclear proteins or protein complexes (named as NF-κB A, B and C) that were already present in untreated cells (Fig. 3A, lanes 1-2), whereas trichodermin exposure alone caused less intense induction of the complexes A and B (Fig 3A, lane 3). Trichodermin and Str. californicus together caused a similar NF-κB complex formation than Str. californicus alone, although complex A was attenuated with the dose of 100 ng/ml (Fig. 3A, compare lanes 2 and 4).

Supershift assays. There are five members of the NF-κB/Rel family of proteins that have been found to be expressed in mammalian cells. These NF-κB/Rel subunits are p65/RelA, c-Rel, Rel B, p105/NF-κB1 (which can be processed to p50), and p100/NF-κB2 (which can be processed to p52). These subunits usually exist as protein hetero- or homodimers. The NF-κB subunits were examined using Abs specific for p65, p50, p52, Rel B, and c-Rel in supershift assays. The Str. californicus inducible complex A was supershifted with the antibody against c-Rel. Antibodies to p65, p50, p52, and Rel B did not have any effect on the complexes (Fig. 3B). Thus other complexes remained unidentified.
Binding of nuclear proteins to \( \kappa BnIL-6 \) oligonucleotide. Exposure to either \textit{Str. californicus} or trichodermin (100 ng/ml) or to both stimuli simultaneously increased the DNA binding activity of complexes NF-\( \kappa B \) A/B and NF-\( \kappa B \) C (Fig. 3C, lanes 1-4). Exposure to higher trichodermin concentration inhibited \textit{Str. californicus} induced DNA binding of complexes NF-\( \kappa B \) A/B and NF-\( \kappa B \) C (Fig. 3 C, compare lanes 2 and 6).

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

**DISCUSSION**

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

Interestingly, although spores of \textit{S. chartarum} alone were not able to evoke any inflammatory responses in macrophages, they triggered a significant, dose-dependent
cytokine response in these cells when exposed in conjunction with a low dose of *Str. californicus*. This is of special interest since it has been shown that the actinomycetes are found together with *Stachybotrys* in 60% of paper materials, indicating that the simultaneous exposure to these microbes in moldy house is a likely scenario (Hyvärinen et al. 2002).

However, co-exposure to fungal and bacterial strains does not invariably evoke inflammatory responses in macrophages since the observed synergistic effect with *Str. californicus* was not seen with the other tested fungal strains, although both *Aspergillus* and *Penicillium* are also among the fungal strains occurring frequently with actinomycetes in moisture and mold damaged building materials (Hyvärinen et al. 2002). To study this phenomenon further, the metabolites typically produced by *S. chartarum* were tested in order to identify the active component(s) of these fungal spores behind the detected synergistic effects with gram-positive bacteria *Str. californicus*. Other fungal metabolites were analyzed for reference purposes, most of them being compounds produced by common indoor air fungal species.

Here we show that the typical metabolites of *S. chartarum* contain at least one compound, trichodermin, which is able to mimic the dose-dependent synergistic inflammatory effects in macrophages as the spores of this fungi. The amplified cytokine responses were triggered in macrophages also by the closely related 7-α-hydroxytrichodermin. Exposure to the actinomycete *Str. californicus* increased the nuclear localization of NF-κB proteins, but the synergistic interaction between trichodermin and actinomycete could not be explained by the amount of NF-κB in the nucleus. Instead, trichodermin may act through a parallel signal transduction pathway leading to the binding of other transcription factors to IL-6 promoter and therefore, enhancing transcription of the IL-6 gene.

These findings are in line with earlier interpretations suggesting that inhalation of fungi, particularly those which produce mycotoxins, results in immunological deregulation. It has been suggested that the exposure to mycotoxins may interfere with the function of
immunologic cells, since already the number of mycotoxins have been shown to activate macrophages in vitro (Ji et al. 1998, Rotter and Oh, 1996, Wong et al. 1998). Moreover, in animal models a low level exposure to the trichotheccenes, ochratoxin and sterigmatocystin has been linked to immune dysfunction and compromised host resistance (Pestka and Bondy 1990).

In the current study, the macrocyclic trichothecene satratoxin G, was highly cytotoxic to exposed cells with and without bacterial exposure. Although the toxicity or inflammatory effect of satratoxin G exposure was not markedly changed by the presence of actinomycete, the toxicity as such is noteworthy since the spores or other particles can act as a carrier of these toxins. The cytotoxic properties of macrocyclic trichotheccenes are well known, thus the tenfold lower effective dose was expected. The atranones B and E, immunosuppressive spirocyclic drimanes staplabin and SMTP-7, nephrotoxic citrinin and carcinogenic sterigmatocystin did not induce any significant production of IL-6 or TNF-α in mouse macrophages, and the hepatotoxic agent, ochratoxin A, caused only a slight increase in TNF-α production in this study. None of these toxins supplemented the inflammatory effect of the spores of *Str. californicus*, on the contrary the TNF-α production was if anything inhibited slightly.

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

A previous chemical study on \textit{Stachybotrys} metabolites revealed that all of the evaluated isolates produced high quantities of the immunosuppressant spirodrimanes, but only one third of the isolates produced the macrocyclic trichotheccenes (mainly satratoxin H and G and roridins L-2 and E). Further studies on the non-macroyclic trichotheccene-producing isolates lead to the isolation of the novel diterpenoids called atranones (Hinkley et al. 2000). The inflammatory responses to atranones have been studied earlier in association with a chemical study of 20 \textit{Stachybotrys} strains and the metabolites they produced. However, the inflammatory effect of non-satratoxin-producing strains could not be attributed exclusively to the atranones (Nielsen et al. 2001). In the present study, trichodermin was not detected by LC-MS in the sample of \textit{S. chartarum}, but the observed effects of exposure to spores could be due to the accumulation of other simple trichotheccenes from this very complex metabolic pathway. This question will remain unresolved until bioguided isolation of the active metabolites has been performed.

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

One of the key elements of our *in vitro* studies has been the development of a relevant model for screening for moldy house exposure, including the interaction between the various possible components of microbial exposure. The synergistic interaction between different exposure agents is a plausible explanation for the adverse health effects already at fairly low microbial concentrations in the indoor air, in addition to the presence of microbes with high inflammatory potentials.

**ACKNOWLEDGEMENTS**

This study was supported by the Academy of Finland, the Finnish Research Programme on Environmental Health (SYTTY) and The Juha Vainio Foundation. The study sponsors had no involvement in the collection, analysis or interpretation of the data, writing the report or submission.

The authors wish to thank Ms. Arja Rönkkö and Ms. Reetta Tiihonen for their excellent technical assistance.

**CONFLICT OF INTEREST STATEMENT**

The authors of this manuscript have no conflicting interests.
REFERENCE


**Figure 1.** (A) The production of IL-6 after exposure to five doses (1-1000 ng/ml) of trichodermin and 7-α-hydroxytrichodermol alone and together with *Str. californicus* (10^5 spores/ml). (B) The production of TNF-α after exposure to five doses (1-1000 ng/ml) of trichodermin and 7-α-hydroxytrichodermol alone and together with *Str. californicus* (10^5 spores/ml). Each column represents mean ± SEM of five independent experiments done in duplicate. C = buffer control; STR = 10^5 spores/ml of *Str. californicus* alone; ( * ) indicates statistically significant difference from control; (+) indicates statistically significant synergistic effect in simultaneous exposure (p<0.05).

**Figure 2.** (A) The cytotoxicity of the exposure to five doses (1-1000 ng/ml) of trichodermin and 7-α-hydroxytrichodermol alone and together with *Str. californicus* (10^5 spores/ml). (B) The cytotoxicity of the exposure to one dose (100 ng/ml) of each microbial metabolites alone and together with *Str. californicus* (10^5 spores/ml). Each column represents mean ± SEM of five independent experiments done in duplicate. Atra E = atranone E; Atra B = atranone B; TriDe = trichodermin; TriDeOH = hydroxytrichodermol; Stapla = staplabin; Satra G = satratoxin G; SteCys = sterigmatocystin; Ochra A = ochratoxin A; STR = 10^5 spores/ml of *Str. californicus* alone; ( * ) indicates statistically significant difference from control; (+) indicates statistically significant synergistic effect in simultaneous exposure (p<0.05).

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

- Trichodermin +
- 7-α-hydroxytrichodermol +

B) Tumor necrosis factor α

- Trichodermin +
- 7-α-hydroxytrichodermol +

- **Streptomyces californicus** (10^5 cells/ml)
- □ Toxin (ng/ml)
- ■ **Streptomyces californicus** (10^5 cells/ml) + toxin (ng/ml)
A) Cytotoxicity of trichodermin and 7-α-hydroxytrichodermol

- Trichodermin +
- 7-α-hydroxytrichodermol +

B) Cytotoxicity of microbial metabolites

- Metabolites of *Stachybotrys*
- Metabolites of other fungi

---

- *Streptomyces californicus* (10⁵ cells/ml) □ Toxin (ng/ml)
- *Streptomyces californicus* (10⁶ cells/ml) + toxin (ng/ml)
A) NF-κB consensus

<table>
<thead>
<tr>
<th>TriDe (ng/ml)</th>
<th>-</th>
<th>100</th>
<th>100</th>
<th>500</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>STR</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

NF-κB A ➪
NF-κB B ➪
NF-κB C ➪

B) NF-κB consensus supershift

<table>
<thead>
<tr>
<th>Antibody</th>
<th>STR</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>p50</td>
<td>p52</td>
<td>p65</td>
<td>Rel B</td>
<td>c-rel</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

supershift ➪
NF-κB A ➪
NF-κB B ➪
NF-κB C ➪
C) NF-κB in IL-6 promoter

<table>
<thead>
<tr>
<th>TriDe (ng/ml)</th>
<th>-</th>
<th>-</th>
<th>100</th>
<th>100</th>
<th>500</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>STR</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

D) PCR fragment of IL-6 promoter

<table>
<thead>
<tr>
<th>TriDe (ng/ml)</th>
<th>-</th>
<th>-</th>
<th>100</th>
<th>100</th>
<th>500</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>STR</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>