BACTERIAL STRAINS FROM MOLDY BUILDING ARE HIGHLY POTENT INDUCERS OF INFLAMMATORY AND CYTOTOXIC EFFECTS

M-R Hirvonen*, K Huttunen, J Jussila, T Murtoniemi, E Iivanainen, H Komulainen, A Nevalainen and M Roponen

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

ABSTRACT

Inflammatory responses and cytotoxicity induced by bacteria, isolated from moldy buildings, were evaluated in human and mouse cells and in a mouse. Human (28SC)- and mouse (RAW264.7) macrophage cell lines were exposed to the spores of *Streptomyces californicus* and bacterial cells of *Mycobacterium terrae* at different doses in vitro and mice (NIH/S) were exposed by intratracheal instillation to the same microbes. The results show that *S. californicus* and *M. terrae* induce production of important inflammatory mediators i.e. nitric oxide (NO) and cytokines both in human and mouse macrophages in vitro, but mouse cells were more sensitive than human cells. In line with in vitro findings, these bacterial strains triggered in vivo time dependent production of the same inflammatory mediators and caused cytotoxicity in mouse lungs. Altogether, these results suggest that in indoor air streptomycetes and mycobacteria are potent inducers of inflammatory responses possibly related to adverse health effects of the inhabitants.

INDEX TERMS

Bacteria, Inflammation, Cytokines, Nitric oxide, Cytotoxicity

INTRODUCTION

The indoor air microbial concentrations in moldy buildings may be only slightly elevated when compared to those in reference buildings, but the detected microbial levels do not necessarily correlate with the reported adverse health effects. Thus, the causal relationship between detected exposures and health end points have been questioned. Based on current epidemiological data, it can be assumed that one of the main contributing factors in the etiological mechanism of the reported symptoms is inflammatory response towards specific organic materials in the microbes. This would include activation of immunological and epithelial cells leading to increased production of cytokines (i.e. interleukins (IL), tumor necrosis factor alpha (TNFα), nitric oxide (NO) and reactive oxygen species (ROS) and subsequent cytotoxicity (Huttunen et al, 2000; Jussila et.al, 2001). These mediators play an important role in regulating pathophysiology of inflammatory diseases including asthma. Moreover, microbes growing on moist building materials are well known to produce toxins. At present there is a serious lack of data based on biochemical evidence of a link between objective biomarkers, qualitative characteristics of the microbial emissions and subjective symptoms. These data are, however, needed for proper risk assessment of the moldy house problem, the measures taken to solve it and assessment of its importance for public health and health economy. The focus of the present studies was on inflammatory responses and cytotoxic effects induced by bacterial strains, i.e. *S. californicus* and *M. terrae*, isolated from moisture- and mold-damaged buildings 1) in human and mouse cells and 2) in a mouse lungs.

* Contact author email: Maija-Riitta.Hirvonen@ktl.fi
Moreover, gram positive *Bacillus cereus* and gram negative *Pseudomonas fluorescens* were used as reference microbes *in vitro*.

**METHODS**

**Bacteria**
The bacteria used in studies were isolated from moldy houses. For identification to species level, the *M. terrae* was analyzed for cellular fatty acid and alcohol composition using gas liquid chromatography. The isolate was tested for growth and biochemical characteristics, and for hybridization with commercial DNA probe. For the experiments, the *S. californicus* was cultured on Trypton yeast-glucose agar, and *M. terrae* on 7H11-agar supplemented with OADC-enrichment, and concentrations were counted under an epifluorescence microscope after acridine orange staining. *S. californicus* was identified in Deutsche Sammlung von Microorganismen und Zellkulturen GmbH and the strains of *B. cereus* and *P. fluorescens* were identified in National Veterinary and Food Research Institute, Kuopio Regional Laboratory, Finland. Other bacterial strains were cultured on trypton yeast glucose agar.

**Cell culture**
A mouse macrophage cell line (RAW264.7) and human macrophage cell line (28SC) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured at 37°C in 5 % CO₂ atmosphere in cell line specific culture medium. The human cells were primed with INF-γ (10 ng/ml) and anti-microbial agents (nystatin and penicillin-streptomycin) were added after the dispensing of the cell. After the exposure to different doses of the bacterial strains for 24 hrs, the adherent cells were resuspended in the culture medium by scraping and the cell suspension was centrifuged (5 min, 8000 rpm). The supernatants and frozen cells were stored at -80°C for the biochemical analyses.

**Experimental animals**
Male NIH/S mice, used in this study, were transferred from the barrier unit to conventional animal room one week before experiments. Mice were exposed dose dependently by intratracheal instillation to a single doses of either the spores of *S. californicus* or *M. terrae* cells. The results by the dose of 10⁸ spores or cells are presented in current paper. Brochoalveolar lavage (BAL) was made at several time points thereafter. The cells were separated from BAL fluid (BALF), and the markers of inflammation and cytotoxicity were measured.

**Biochemical analyses**
The methods used are listed in Table 1.

**Table 1. Analyses in cell culture studies and in animal studies**

<table>
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<tr>
<th>Parameters to be analyzed</th>
<th>Pathophysiol. functions</th>
<th>Methods</th>
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<td>Nitric oxide (NO): nitrite/inducible NO-synthase (iNOS)</td>
<td>-vasodilation, edema, cytotoxicity -initiation of inflammation respiratory diseases (asthma)</td>
<td>Griess/Western Blot (Hirvonen et al. 1996)</td>
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<td>Cytokines:</td>
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<td>IL-6, TNFα</td>
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RESULTS

In vitro studies

Production of inflammatory mediators

Mouse RAW264.7 macrophages produced large amount of TNF-alpha after exposure to B. cereus (up to 3900 ± 700 pg/ml), S. californicus (up to 6100 ± 880 pg/ml), M. terrae (up to 1400 ± 680 pg/ml) and P. fluorescens (up to 8000 ± 1100 pg/ml). None of the studied microbes caused any significant production of TNF-α in human 28SC macrophages. P. fluorescens was a highly potent inducer of the production of IL-6 both in mouse macrophages (up to 2370 ± 30 pg/ml) and in human 28SC macrophages (up to 480 ± 180 pg/ml). Interestingly, M. terrae and S. californicus induced a strong IL-6 response in the mouse macrophages (up to 2150 ± 40 pg/ml), and a clear difference compared to control cells also in human macrophages although the cytokine levels were much lower (up to 170 ± 70 pg/ml). B. cereus induced mouse macrophages to produce somewhat increased amounts of IL-6 (up to 400 ± 340 pg/ml) when compared to controls after 24 hours of exposure and no significant response in human cell lines. P. fluorescens induced the strongest NO-responses, reaching as high as 28,6 ± 1,6 µM. Exposure to S. californicus and M. terrae resulted in almost as high concentrations of nitrate in cell culture media (up to 28,3 ± 1,6 µM). B. cereus did not cause significant NO production. Human macrophages produced NO only after the exposure to S. californicus and M. terrae, but the induced concentrations were much lower when compared to those in mouse macrophages.

Cytotoxicity

The viability of mouse macrophages decreased after exposure to bacteria, which induced also the production of inflammatory cytokines. S. californicus and P. fluorescens were most toxic to these cells when compared to controls, increasing the cytotoxicity up to 76 ± 2 and 70 ± 3%, respectively, followed by B. cereus which was cytotoxic to up to 74 ± 4 % of the macrophages. Exposure to M. terrae was not markedly cytotoxic. All the studied bacteria were less toxic to the human macrophage cell line, cytotoxicity caused by the exposure ranging from 21 ± 15 to 28 ± 6 % when compared to control cells.

In vivo studies

Production of inflammatory mediators

The exposure to the spores of S. californicus as well as M. terrae cells increased TNFα and IL-6 production, and induced expression of iNOS in mouse lungs. TNFα concentration peaked at 6 hours in both experiments, and the concentrations were 13- and 11-fold when compared to the controls after the instillation of the spores or mycobacterial cells, respectively. During the spore exposure the TNFα concentrations decreased to the control level by 3 days, but the mycobacterium provoked sustained response which did not leveled off until 21 days. IL-6 concentrations peaked at the same time as TNFα concentrations after the instillation of either the spores or the mycobacterium cells. However, the spores of S. californicus induced approximately 3 and 7 fold higher IL-6 response than M. terrae at 6 and 24 hours, respectively. Moreover, IL-6 responses leveled off by 3 days. The spores of S. californicus induced the expression of iNOS transiently at 24 hours, whereas mycobacterial cells induced sustained expression of the enzyme which was first time detected at 7 days after the instillation and the response lasted up to 28 days.

Cytotoxicity

Cytotoxicity in lungs was evaluated by measuring intracellular enzyme, lactate dehydrogenase (LDH) leakage to the airways. The studied dose of the spores of S. californicus doubled LDH
concentration in BALF compared to the control at 24 hours and this difference was statistically significant. *M. terrae* increased LDH concentration statistically significantly at 24 hours (144% above the control level), but also at 14 and 21 days (142% and 140% above the control level, respectively), indicating sustained cytotoxicity due to mycobacterial exposure.

**DISCUSSION**

The current data clearly show that streptomycetes and mycobacteria isolated from moldy buildings are highly potent to trigger the production of important inflammatory mediators, such as NO and cytokines (i.e. TNFα, IL-6) in mouse macrophages and induce production also in human macrophages. In line with these *in vitro* findings a single dose of the spores of *S. californicus* and *M. terrae* cells provoked an acute production of, as a rule, the same mediators in mouse lungs. Moreover, *M. terrae* exposure induced also some sustained effects. These results are of interest since it has been hypothesized that stimulation of macrophages to produce TNFα is a common mechanism for particles to trigger inflammation in lungs (Driscoll et al. 1997). TNFα is then supposed to stimulate production of other inflammatory mediators via autocrine and paracrine pathways, for example that of other cytokines such as IL-6, chemokines and NO. Moreover, the induced NO production is thought to be an important factor in acute lung injury (Kristof et al., 1998) and sustained high concentrations of NO may cause vasodilatation, edema and cytotoxicity (Clancy and Abramson, 1995; Barnes et al., 1998). IL-6 has been reported to be involved in lymphocyte activation, growth and differentiation, but may also reduce TNFα production and neutrophil influx into the airways (Barnes et al., 1998).

Induced production of cytokines by the spores of *S. californicus* was associated with cytotoxicity, whereas the exposure to the cells of *M. terrae* was nontoxic. Observed cytotoxicity in mouse lungs was chronologically associated with the production of inflammatory mediators. Pure particle effect of the bacterial cells or spores is unlike, because equal doses of similar sized and shaped bacteria *B. cereus* caused only slight inflammatory responses. This is also in line with our earlier reports demonstrating that the potency of different species of streptomycetes to stimulate NO and reactive oxygen species production, and to cause cytotoxicity varies significantly (Hirvonen et al., 1997). Thus, the current data show that the studied bacterial strains differ significantly from each other in potency, time-course, and induced spectrum of inflammatory mediators both *in vitro* and *in vivo*. Moreover, these results show that there is a good correlation between the *in vitro* and *in vivo* data.

**CONCLUSIONS AND IMPLICATIONS**

Based on our present findings, following insights can be gained: 1. Streptomycetes and environmental mycobacteria are highly potent inducers of inflammatory responses in human and mouse cells *in vitro*. However, the human and mouse cell lines differ in their ability to be activated by these microbes; mouse RAW264.7 macrophages being more sensitive than human 28SC cells. 2. In line with *in vitro* findings, these bacterial strains cause an inflammation in mouse lungs, indicated by increased production of the inflammatory mediators.

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REFERENCES