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Interactions between *Streptomyces californicus* and *Stachybotrys chartarum* can induce apoptosis and cell cycle arrest in mouse RAW264.7 macrophages

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Abstract

Exposure to complex mixtures of bacteria and fungi in moisture-damaged buildings is a potential cause of inflammatory related symptoms among occupants. The present study assessed interactions between two characteristic moldy house microbes *Streptomyces californicus* and *Stachybotrys chartarum*. Differences in cytotoxic and inflammatory responses in mouse (RAW264.7) macrophages were studied after exposure to the spores of co-cultivated microbes, the mixture of separately cultivated spores, and the spores of either of these microbes cultivated alone. The RAW264.7 cells were exposed to six doses (1×10^4 to 3×10^6 spores/ml) for 24 h, and the time course of the induced responses was evaluated after 4, 8, 16, and 24 h of exposure (1×10^6 spores/ml). The cytotoxic potential of the spores was characterized by the MTT test, DNA content analysis, and enzyme assay for caspase-3 activity. The production of cytokines (IL-1β, IL-6, IL-10, TNFα, and MIP2) was measured immunochemically and nitric oxide by the Griess method. Co-cultivation increased the ability of the spores to cause apoptosis by more than 4-fold and the proportion of RAW264.7 cells at the G_2/M stage increased nearly 2-fold when compared to the response induced by the mixture of spores. In contrast, co-cultivation decreased significantly the ability of the spores to trigger the production of NO and IL-6 in RAW264.7 cells. In conclusion, these data suggest that co-culture of *S. californicus* and *S. chartarum* can result in microbial interactions that significantly potentiate the ability of the spores to cause apoptosis and cell cycle arrest in mammalian cells.

Keywords: Apoptosis; Cell cycle arrest; Interaction; Co-cultivation; RAW264.7; *Streptomyces californicus*; *Stachybotrys chartarum*

Introduction

Though there is convincing worldwide epidemiological data (Bornehag et al., 2001; Husman, 1996; Peat et al., 1998), actual causal relationships between human exposure in moisture and mold damaged buildings and human health have been difficult to establish. Understanding this relationship has proved elusive because the interactions occurring between different exposures in a moisture-damaged building are inevitably due to a wide variety of pollutants, including many microbial species (Hyvärinen et al., 2002). Potential toxin-producing fungi such as *Stachybotrys* spp., *Aspergillus* spp., *Fusarium* spp., and actinobacteria *Streptomyces* spp. are among the microbes frequently present in such environments (Samson et al., 1994). Different growth conditions provided by building materials and competition between microorganisms for the same habitat may change the bioactivity of the microbes and their spores (Murtoniemi et al., 2003). Previous results support this view, because co-cultivation

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of various microorganisms has been shown to alter protein expression (Meyer and Stahl, 2003) and reduce toxin production of the spores (Ehrlich, 1987).

At present, little is known about the interactions between different moldy house microbes, although there are some reports on inflammatory and cytotoxic potential of certain microbes and microbial components (Huttunen et al., 2003; Fogelmark et al., 2001; Jagielo et al., 1996). Our earlier studies indicate that compared to fungi, actinobacteria are more potent at inducing the production of inflammatory mediators both in mouse and human cell lines in vitro (Huttunen et al., 2003) and in mouse lungs in vivo (Jussila et al., 2001, 2002). In addition, our recent results show that, of the six studied microbial strains, only *Stachybotrys chartarum* and fungal metabolite trichodermin are able to potentiate the inflammatory response of actinobacteria *Streptomyces californicus* (Huttunen et al., 2004). The spores of *S. californicus* are able to induce the nuclear binding activity of well-known transcription factor NF-κβ in RAW264.7 cells, but the synergistic interaction between actinomycete and trichodermin cannot be explaned by amount of NF-κβ in the nucleus (Huttunen et al., 2004). Thus, the exact mechanisms of inflammation and cytotoxicity triggered by these microbial spores are still obscure.

Previous results demonstrate that trichothecenes produced by *Stachybotrys* spp. induce apoptosis, which can proceed through both the mitochondrial pathway and the death receptor pathway (Nasage et al., 2002; Yang et al., 2000). In the mitochondrial pathway a loss of the mitochondrial membrane potential increases permeability of its outer membrane, resulting in the release of cytochrome *c* and other apoptosis-promoting factors such as Smac/Diablo (Hengartner, 2000). The released cytochrome *c* interacts with apoptotic protease activating factor-1 (Apaf-1), pro-caspase-9, and dATP to form a complex, which activates caspase-9 and subsequently other caspases, including caspase-3 (Li et al., 1997). Smac/Diablo promotes caspase-9 activation by binding to inhibitor apoptosis proteins (IAPs) and thus removing their inhibitory activity (Du et al., 2000). The other apoptosis pathway induced by death receptors of the tumor necrosis factor (TNF) family such as Fas, TNFR1, or TRAIL receptors leads to activation of caspase-8 after the adaptor protein FADD binds to its receptor (Hengartner, 2000; Thorburn, 2004). Caspase-8 subsequently activates effector caspases such as caspase-3, which is thought to be responsible for cellular autoproteolysis and DNA fragmentation.

Pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) is a well-characterized mediator that modulates cysteine protease activation and the production of nitric oxide (NO), and this can trigger *mycobacterium*-induced macrophages to undergo caspase-mediated apoptosis (Rojas et al., 1999). NO is a potent mediator that is capable of inducing DNA damage leading to apoptosis in macrophages (Meßmer et al., 1994). NO can also induce cell cycle arrest before mitosis in the early G_2/M phase in the presence of interleukin-6 (Takagi et al., 1994). Although apoptosis is considered to be a controlled and regulated form of cell death, inflammation is not necessary totally absent in the apoptotic process, because previous results indicate that cells undergoing apoptosis can release pro-inflammatory cytokines into their immediate environment (Hogquist et al., 1991).

The aim of the study was to assess the interactions between the spores of co-cultivated Gram-positive bacterial strain *S. californicus* and the fungal strain *S. chartarum*. The differences in cytotoxicity and inflammatory responses in mouse (RAW264.7) macrophages were detected after exposure to the spores of co-cultivated microbes and the respective mixture of spores and the spores of either of these microbes alone.

Methods

Microbial strains. S. californicus was isolated from the indoor air of a building with moisture problems using a six-stage impactor and tryptone yeast-glucose agar (TYG; Bacto Plate Count Agar, Difco Laboratories, Detroit, MI, USA). *S. chartarum* was isolated from a moisture-damaged building material sample on 2% malt extract agar (MEA; Biokar Diagnostics, Beuvais, France). The identification of *S. californicus* was confirmed by the DSM identification service (DSMZ-Deutsche Sammlung von Microorganismen and Zellkulturen, Germany) and *S. chartarum* was confirmed by the CBS identification service (Centraal Bureau of Schimmelcultures, Utrecht, the Netherlands). The microbial strains were stored at −20 °C until the experiments. Both strains were cultivated separately on 2% MEA as a dense culture. In addition, *S. californicus* was co-cultivated with *S. chartarum* on the same plate inoculated in the proportion of 100:1 (*S. californicus*: *S. chartarum*). The plates were incubated at 25 °C in the dark until the microbes sporulated (17–40 days). After incubation, the spores were collected with a sterile loop and suspended in Hank's balanced salt solution (HBSS) (Gibco, Paisley, UK) containing 0.0001% Triton X-100. At the end of the co-cultivation, the spore concentrations and proportion of the microbes were determined by counting the spores after acridine orange staining using an epifluorescence microscope. The mixture of the spores of separately cultivated *S. californicus* and *S. chartarum* was prepared at the same proportion (5:1) as counted at the end of the co-cultivation of these microbes.

Cell culture. The mouse macrophage cell line RAW264.7 was obtained from the American Type Culture Collection (ATTC, Rockville, MD, USA). The cells were cultured at 37 °C in 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% of heat-inactivated fetal bovine serum (FBS), L-glutamine (2 mM), and penicillin–streptomycin (100 U/ml) (all from Gibco). The RAW264.7 cells