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Inflammatory Cytokine Gene Expression in THP-1 Cells Exposed to *Stachybotrys chartarum* and *Aspergillus versicolor*

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Abstract

Very little is known about the mechanisms which occur in human cells upon exposure to fungi as well as their mycotoxins. A better understanding of toxin-regulated gene expression would be helpful to identify safe levels of exposure and could eventually be the basis for establishing guidelines for remediation scenarios following a water intrusion event. In this research, cytokine mRNA expression patterns were investigated in the human monocytic THP-1 cell line exposed to fungal extracts of various fragment sizes obtained from *Stachybotrys chartarum* RTI 5802 and/or *Aspergillus versicolor* RTI 3843, two common and well studied mycotoxin producing fungi. Cytokine mRNA expression was generally upregulated 2 to 10 times following a 24-hour exposure to fungal extracts. Expression of the proinflammatory interleukin-1 β (*IL-1 β*), Interleukin-8 (*IL-8*) and tumor necrosis factor- α (*TNF α*) genes increased while the anti-inflammatory gene Interleukin-10 (*IL-10*) also increased albeit at very low level, suggesting that negative feedback regulation mechanism of production of pro-inflammatory cytokines initiated upon 24 hours of incubation. In addition, submicron size extracts of *A. versicolor* caused significant death of THP-1 cells whereas extracts of *S. chartarum* caused no cell death while the mixture of the two fungi had an intermediate effect. There was no general correlation between gene expression and fragment sizes, which suggests that all submicron fragments may contribute to inflammatory response.

Keywords

Mycotoxin, Cytokine; THP-1; *Stachybotrys chartarum*; *Aspergillus versicolor*

INTRODUCTION

The presence of pathogenic fungi in indoor environments has been linked to adverse health consequences resulting from the production of fungal toxins (Pestka et al. 2008; Ueno 1985). *Stachybotrys* and *Aspergillus* spp. are both well studied fungi which are commonly found in flooded buildings. These fungi are often associated with the production of a wide range of mycotoxins. *Stachybotrys chartarum* is best known for producing highly toxic trichothecenes such as verrucarins B and J, roridin E, satratoxins J, G and H, and isosatratoxins F, G and H (Brasel et al. 2005b; Hinkley and Jarvis 2001; Jarvis et al. 1998). There have also been reports of *Stachybotrys* spp. producing spirocyclic drimanes and diterpenoid atranones (Fog Nielsen 2003). *Aspergillus versicolor* is mainly known for producing sterigmatocystin, a carcinogenic mycotoxin (Fog Nielsen 2003). However, other

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mycotoxins including ocratoxin A and trichothecenes such as nivalenol and deoxynivalenol have also been detected in some *Aspergillus* spp. (Atalla et al. 2003; Atoui et al. 2007; El-Shanawany et al. 2005). In general, trichothecenes are viewed as some of the most potent mycotoxins because they are known to be extremely toxic to leukocytes and other rapidly dividing cells. Trichothecenes are also known to contribute to several human diseases including alimentary toxic aleukia (*Fusarium* spp), stachybotryotoxicosis (*Stachybotrys* spp.) and red mold disease (*Fusarium* spp.) (Sidell et al. 1997). For these reasons, trichothecenes are commonly measured in fungal analyses. However, even though adverse health effects are known to occur following exposure to fungi, it is unclear what the impacts of mycotoxin mixtures found on fungal fragments and spores in general are at the molecular level in human cells.

To date, most research has focused on epidemiological data and suggests a link between exposure to molds in damp indoor environments and upper and lower respiratory illnesses (Flemming et al. 2004; Mason et al. 2001; Wilkins et al. 1998) as well as cancer (Anyanwu et al. 2002). However it is unclear what specific genes are affected by mycotoxins in humans. It is also unclear what concentrations and which fungal particle sizes play the most critical role in eliciting the response. In addition, an understanding of the cellular response resulting from such exposure is one crucial mechanism that has not been well studied and could serve as a basis for establishing guidelines for setting safe exposure limit and rebuilding/remediation scenarios. The present study was undertaken to ascertain the effect of mycotoxin mixtures found in the fungal extracts of *Stachybotrys chartarum* RTI 5802 and *Aspergillus versicolor* RTI 3843. Specifically, the effect of submicron fragments of varying sizes on the expression of inflammatory cytokine genes was measured. Gene expression patterns were investigated in the human monocytic cell line THP-1, a cell line used widely to investigate the regulation of gene expression of inflammatory cytokines by various disease-causing agents (Boutten et al. 1992; Broaders et al. 2006; Ellertsen et al. 2006; Fenton et al. 1987; Gatanaga et al. 1991; Krakauer and Oppenheim 1983; Tsuchiya et al. 1980). To the authors' knowledge, this is one of the first studies addressing mold- and/or mycotoxin-regulated inflammatory gene expression in human monocytic THP-1 cells. Most other studies have relied on the mouse monocytic RAW264.7 cell line (Chung et al. 2003a; Chung et al. 2003b; Mbandi and Pestka 2006; Moon and Pestka 2002; Pestka and Zhou 2006) and the human A549 lung epithelial cell line (Instanes and Hetland 2004; Johannessen et al. 2007). Given the report that mycotoxins can be toxic to leukocytes and cause human and animal immune diseases (Joffe and Yagen 1977), the human monocytic THP-1 cell line is physiologically relevant to this study. Genes for proinflammatory cytokines *IL-1 β* , *IL-6*, *IL-8*, and *TNF α* as well as anti-inflammatory cytokine *IL-10* were targeted for this study. These genes have been shown to be key mediators in regulating immune and inflammation response to microbes, toxins, trauma, and ischemia (Dinarello 2000).

MATERIALS AND METHODS

Fungal Strains and Culture Conditions

S. chartarum RTI 5802 and *A. versicolor* RTI 3843 were used as the parent strains for all experiments. Both fungal species were cultured on sporulating agar medium (0.5 g/L dextrose, 10 g/L potassium acetate, 1 g/L yeast extract and 20 g/L agar) at room temperature as described (Gunsch et al. 2005). Stocks of each strain were maintained in 10% glycerol at -80°C for long term preservation. New cultures were derived from the frozen stock at the beginning of each experiment.

Aerosolization Chamber

A laboratory scale reactor was constructed and used as an aerosolization chamber to collect fungal fragments for subsequent use in the exposure experiments (Figure S1). The reactor consisted of a single cylindrical acrylic compartment 19 cm in diameter and 20 cm in height. The reactor was sealed with a hinged lid and a rubber ring. Moist room temperature air was pulled through the reactor using a 23 series oil-less vacuum pump (GAST, Benton Harbor, MI). Inlet and outlet valves were located 7 and 10 cm, respectively, from the bottom of the reactor. The vacuum pump and sampling device were connected to the reactor using Tygon® tubing of various diameters. To maintain moisture in the chamber, the air stream was fed through a nebulizer (Vortran Medical Technology, Sacramento, CA) filled with deionized water. Petri dishes with thick fungal mats of *S. chartarum* RTI 5802 and/or *A. versicolor* RTI 3843 were placed on the bottom of the chamber at the beginning of each experimental phase. As air was blown into the chamber, fungal fragments were collected using a 4-stage Sioutas cascade impactor (SKC, Eighty Four, PA). The Sioutas cascade impactor was used to separate and collect particles in five size groups (<0.25, 0.25-0.5, 0.5-1.0, 1.0-2.5 and >2.5 µm). Each collection stage was lined with aluminum foil to facilitate extraction. Samples for each individual fungus as well as for both fungi simultaneously were collected in triplicate. For each experiment, a total of 2 Petri dishes were placed in the chamber. Each sampling period lasted seven days.

Fungal Fragment Extraction

Following collection, size differentiated fungal fragments were extracted for subsequent use in toxicity assays. Extraction was carried out only if a measurable mass of fungal fragment was obtained for a given size range. Briefly, fragments were rinsed off of the collection stage using 2 mL methanol and placed into a 25 mL glass vial. Ten mL sterile deionized water was added to the wash solution and the vial was stored at -80 °C overnight. Each sample was then freeze dried. Finally, the fungal extracts were resuspended in sterile deionized water and dissolved by sonication. All fungal extracts were stored at -20 °C for further use and/or analysis. The concentration of the trichothecenes in the fungal extracts was quantified using a QuantiTox™ ELISA kit (Envirologix, Portland, ME) according to the manufacturer's instructions.

THP-1 Cell Cultivation

The human monocytic cell line THP-1 was obtained from Dr. Leslie Parise at the University of North Carolina (Chapel Hill, NC). Cells were maintained at 37°C in a 5% CO₂ humidified incubator in RPMI 1640 growth medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Incubations were maintained in either 6- or 96-well cell culture plates with flat bottoms (Fisher Scientific, Pittsburgh, PA) for subsequent mRNA extraction or MTT assay, respectively. For each treatment (*S. chartarum* RTI 5802 alone, *A. versicolor* RTI 3843 alone, and mixture of *S. chartarum* RTI 5802 and *A. versicolor* RTI 3843), THP-1 cells (5.5×10^5 cells/ml) were incubated for 24 hours in the presence of the fungal extracts collected from the three different treatments as described above and assayed for cytokine expression and cell viability. Cells not exposed to fungal extracts were used as the negative control while cells exposed to 1 ng/mL roridin A were used as the positive control. All control experiments were performed in triplicates in parallel with all other treatments.

MTT Assay

The MTT assay was utilized to measure cell viability and was performed as previously described (Carmichael et al. 1987). Briefly, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5,-diphenyltetrazolium bromide) (Sigma, St. Louis, MO) was dissolved in 0.01 M phosphate-

buffered saline (PBS) at pH 7.4 at a final concentration of 5 mg/mL. THP-1 cells (5.5×10^5 cells/mL) were incubated with each fungal extract of 100 μ L in a 96-well tissue culture plate. The total final volume was 200 μ L including both the cell suspension and the fungal extract. Following an incubation period of 24 hours, MTT reagent (20 μ L/well) was added and the plate was incubated for an additional 3 hours. The cells were then washed three times in PBS. Finally, MTT formazan crystals were dissolved in 200 μ L of dimethyl sulfoxide (DMSO), and the absorbance was measured on a microplate reader (Thermo, Vantaa, Finland) at 540 nm.

RNA extraction

Total RNA extraction was performed on each sample using RNAqueous® kits (Ambion, Austin, TX) according to the manufacturer's protocol. The final elution volume used was 70 L. RNA concentration and quality were verified by spectrophotometric analysis using an ND-1000 spectrophotometer (Nanodrop, Wilmington, DE). Only samples with A_{260}/A_{280} ratio greater than 1.8 were used. RNA samples were stored at -80°C for further analysis.

cDNA synthesis

cDNA was synthesized using Applied Biosystems High-capacity cDNA Reverse Transcription kits (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. The following mixture was used for the cDNA synthesis step: 0.13 μ g – 0.5 μ g total RNA, 2 L 10X reverse transcriptase buffer, 4 mM dNTPs, 2 L 10X reverse transcriptase random primer, 1 L MultiScribe reverse transcriptase, and 1 L RNase inhibitor. The reaction was carried out in a 20 L volume using an Applied Biosystem 2720 PCR Thermal Cycler (Foster City, CA).

Gene expression analysis

Gene transcript numbers were quantified using a Stratagene Mx3000P Real Time PCR apparatus (Stratagene, La Jolla, CA). Since SYBR Green I can bind non-specifically to all double-stranded DNA, optimization steps were performed to eliminate signals obtained from either primer-dimer complexes or other non-specific products. Five target genes were monitored using published primer sequences and included the interleukin genes *IL-1 β* (Baqui et al. 1998), *IL-8* (Vitiello et al. 2004), and *IL-10* (Usui et al. 2004); Tumor necrosis factor TNF α (Rosenbaum et al. 1995), and the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) (Jiang et al. 2004). Expression of proinflammatory cytokines *IL-1 β* , *IL-8*, *IL-10*, and *TNF α* were normalized to the expression of GAPDH. Each reaction was carried out in a 25 μ L mixture consisting of 12.5 μ L 1 $\times$ iTaq SYBR Green Supermix with ROX (Biorad, Hercules, CA), 0.2 μ M of reverse and forward primer, and 1 μ L of cDNA template. The thermal cycler program consisted of an initial denaturing step at 95°C for 2 min, followed by 50 cycles of 95°C for 30 or 60 s, 60°C for *IL-1 β* [55°C for *IL-8* and *IL-10*, 56°C for *TNF α* , 55°C for GAPDH for 30 to 60 s, 72°C for 30 or 60 s (optical window on)], and using the default settings for the melting curve stage.

Statistical Analysis

For all experiments, standard deviations were calculated and are included in the figures. Statistical significance was determined by performing a paired student t-test comparing transcript numbers between the negative control (no fungal extract) and each treatment. The analysis was carried out using Microsoft Excel (Seattle, WA) using a 95% significance interval.

RESULTS

Trichothecene Production by Fungal Fragments

In order to determine the levels of trichothecenes that *S. chartarum* RTI 5802 and *A. versicolor* RTI 3843 produced under the experimental growth conditions, the QuantiTox™ ELISA kit which is specifically designed to measure trichothecenes levels was used. This kit which has been used in other published studies (Brasel et al. 2005a; Charpin-Kadouch et al. 2006; Sudakin and Fallah 2008) is designed for the quantitative detection of a variety of trichothecenes including roridin A, E, H and L-2, satratoxin G and H, isosatratoxin F, verrucarol A and J, and verrucarol. While this kit does not allow for the specific identification of mixture components, it has been shown to accurately provide a composite measure of trichothecenes in a given sample. In general, the trichothecene concentrations in the fungal extracts were low. In the 0.25-0.5 μm fragment size range, the average trichothecene concentrations were 0.2, 0.2 and 0.3 ppb for the *S. chartarum* RTI 5802, *A. versicolor* RTI 3843 and fungal mixture, respectively. In the 0.5-1 μm size range, the trichothecene concentrations were consistently found to be below the detection limit of the QuantiTox™ ELISA kit (0.14 ppb). Fragments of other sizes (i.e., <0.25, 1-2.5, or >2.5 μm) which were recovered from the cascade impactor had trichothecene levels consistently below the ELISA kit detection limit as well as below the measuring accuracy of our scale for a weight estimate. Thus, these fragment sizes were not included in subsequent analyses.

Effects of Submicron Fragment Exposure on Cytokine Expression in THP-1 cells

Human monocytic THP-1 cells were incubated with the two recoverable submicron fragment fractions (0.25-0.5 and 0.5-1.0 μm) from *S. chartarum* RTI 5802 alone, *A. versicolor* RTI 3843 alone, or a mixture of both fungi. Both size ranges of *S. chartarum* RTI 5802 fungal fragments were found to upregulate the mRNA expression of all proinflammatory cytokines significantly. The upregulation varied from 2 to 9 fold as compared to the background gene expression ($p < 0.05$) (Figure 1). Fragments from *A. versicolor* RTI 3843 of size 0.25-0.5 and 0.5-1.0 μm upregulated *TNFA* by 11.6 and 8.9-fold, respectively. However, significant upregulation (9.6 fold) was only observed for *IL-1 β* when cells were exposed to the 0.25-0.5 μm fragments, and for *IL-8* (2.1 fold) and *IL-10* (4.8 fold) in the presence of the 0.5-1.0 μm fragments (Figure 2). The degree of cytokine upregulation was significantly different dependent on the fragment size and, thus, these data suggest that fungal fragment size may play a role in the regulation of cytokines for *A. versicolor* RTI 3843 (Figure 2). Exposure to submicron fragments from the fungal mixture was associated with upregulation (2 to 9-fold) of all proinflammatory cytokine mRNA for both size distribution (Figure 3).

Cell Viability Following Fungal Fragment Exposure

Cell viability as measured by the MTT assay, which provides a measure of cellular reductase activity, demonstrated a much stronger effect by *A. versicolor* RTI 3843 than *S. chartarum* RTI 5802. There was no observable effect on cell viability with *S. chartarum* fungal extracts of either size range (Figure 4). In contrast, viability of THP-1 cells exposed to *A. versicolor* fungal extracts was significantly impacted. Viable cells only represented $7.3 \pm 1.3\%$ of the total cells following exposure to the 0.5-1 μm fragment sizes and $61.8 \pm 27.9\%$ for the 0.25-0.5 μm size range. The fungal mixture had an intermediary response with $64.7 \pm 9.7\%$ viable cells with 0.5-1 μm size and $76.6 \pm 37.9\%$ for the 0.25-0.5 μm size (Figure 4). Interestingly, the toxic effect of the 0.5-1.0 μm fragments obtained from *A. versicolor* RTI 3843 decreased in the mixture ($7.3 \pm 1.3\%$ *A. versicolor* alone vs. $64.7 \pm 9.7\%$ in the mixture) indicating the importance of looking at mixtures versus single fungus exposure scenarios. THP-1 cells exposed to 1 ng/ml Roridin A (i.e., positive control cells) were statistically indistinguishable between the different experiments ($p = 0.74$). Viability of THP-1 cells

exposed to extracts from the fungal mixture were also statistically indistinguishable from that exposed to 1 ng/ml roridin A ($p = 0.56$ at 0.25-0.5 μm size; $p = 0.57$ at 0.5-1.0 μm size). However, the viability of THP-1 cells exposed to *A. versicolor* RTI 3843 fungal extracts from the 0.5-1.0 μm size range were significantly different from the positive control ($p < 0.001$).

DISCUSSION

Physiological Relevance of Cell Lines

While there are a large number of published reports on mycotoxin-induced cytokine gene expression in murine cells lines (e.g., RAW 264.7) (Bae and Pestka 2008; Chung et al. 2003; Wong et al. 2001; Wong et al. 1998), very few have reported the effects of mycotoxins in human cell lines such as the monocytic THP-1 cells used in the present study. Shalit et al. (Shalit et al. 2006) reported that extracts of *Aspergillus fumigatus* caused an increased expression of cytokines including *IL-1 β* , *IL-8* and *TNF α* in THP-1 cells. Heller et al. (2002) also showed that extract of *Aspergillus ochraceus* as well as pure mycotoxin ocratoxin A affected cytokine *TNF α* expression in THP-1 cells. The findings herein demonstrate that *A. versicolor* RTI 3843 and *S. chartarum* RTI 5802 can also induce expression of cytokines, further confirming that significant inflammatory response can be expected when humans are exposed to the mycotoxins found in fungal extracts. The amount of upregulation, however, is specific to the fungal strains as well as the particle size itself.

Fungal Fragment Size Effect

Most indoor air quality studies with *A. versicolor* and *S. chartarum* have focused on airborne fungal spores, with size between 3 and 10 μm (Gorny et al. 2002; Li et al. 2002), even though recently published studies suggest that submicron ($< 1 \mu\text{m}$) fungal fragments play a more important role in allergic response and overall respiratory illness (Green et al. 2006). Submicron fragments can easily carry mycotoxins to a receptor and have different aerosolization properties than larger fragments. This finding is of particular importance because of the difficulty of collecting and identifying fungal fragments from contaminated environments in the submicron category. Because submicron fragment concentrations are believed to be 100-500 times higher than spore concentrations (Gorny et al. 2002), it is critical that the toxicological effect of submicron fragments be studied. Furthermore, if submicron fragments are the largest contributor to mycotoxin-induced respiratory illness, this could explain why no dose response has been established linking illness rate to spore levels. The present study offers additional evidence on the health consequences of submicron fragments and provides new information as to the size effect of fungal fragments on inflammatory response.

Traditionally, two approaches have been utilized to isolate the effect of different fungal fragment sizes: aerosolization chambers (Gorny 2004) or the fungal spore source strength test (Sivasubramani et al. 2004a; Sivasubramani et al. 2004b). In both cases the fungal fragments are released by blowing clean air over a fungal growth surface using a controlled flow rate (Gorny 2004). In the present study, an aerosolization chamber was used in series with a cascade impactor enabling the separation and collection of fungal particles in five size ranges (<0.25 , 0.25-0.5, 0.5-1.0, 1.0-2.5 and $>2.5 \mu\text{m}$). The aerosolization chamber approach is significantly different than the fungal spore source strength test which shows that the fungal fragment toxicity and adverse health effects are correlated to the presence of (1 $\rightarrow$ 3)- β -D-glucan in fungal fragments (Seo et al. 2008; Seo et al. 2009). Because of the differences in these methods, it is difficult to draw analogies between the present research results and those reports, especially since the fungal fragments were pooled in different size ranges in our study than those used in fungal spore source strength test.

Mechanisms of Trichothecene-induced Responses

While the exact mycotoxin composition in *A. versicolor* RTI 3843 and *S. chartarum* RTI 5802 extracts is unknown, the ELISA measurements suggest that trichothecene levels were most significant in submicron fragments. Trichothecenes were measured over other mycotoxins because they are an important class of mycotoxins which are known to cause diseases such as alimentary toxic aleukia and pulmonary hemorrhage (Sudakin 2003), are known to be teratogenic and carcinogenic (Schoental 1983) and have been shown to inhibit protein synthesis in most eukaryotic cells (Cole and Cox 1981).

The alteration of cytokine gene expression in THP-1 cells by pure trichothecenes (e.g., deoxynivalenol) and extract of *Aspergillus fumigatus* has been reported to be mediated by Erk1/2, p38 mitogen-activated kinase (Shalit et al. 2006), PKC (Ma et al. 2001; O'Sullivan et al. 2008) and the Toll like receptor 4 (Wu et al. 2008). Others have reported the involvement of Bruton's tyrosine kinase in the cytosol (Jefferies et al. 2003), which in turn activates downstream transcriptional factors NF κ B, SP1, STAT-1 and AP-1 in the cell nuclei (Chen et al. 2002; Hall et al. 1999; Jefferies et al. 2003; Ma et al. 2001). While it is clear in the present study that the mycotoxins in the fungal extracts affected the expression of *IL-1 β* , *IL-8*, *IL-10* and *TNF α* (Figure 1, 2 and 3), the specific mechanisms of induction remain unclear. Follow up experiments are needed to further elucidate the pathways through which fungal extracts induce cytokine gene expression in order to develop a better understanding of the mechanisms of mycotoxin toxicity and to determine if the observed upregulation is mediated through similar pathways as in *A. fumigatus*.

Expression of pro-inflammatory cytokine genes *IL-1 β* , *IL-8* and *TNF α* increased while expression of anti-inflammatory cytokine gene *IL-10* increased at a much lower level (Figure 1, 2 and 3). Feedback of cytokines onto the cells that synthesize them and crosstalk among the various cytokines has been reported. For example, anti-inflammatory cytokine IL-10 has been shown to inhibit the expression of pro-inflammatory cytokine genes including *IL-1 β* and *TNF α* in THP-1 cells (Murthy et al. 2000). Our results suggest that negative feedback regulation mechanism of production of pro-inflammatory cytokines (IL-1 β , IL-8 and TNF α) by the anti-inflammatory IL-10 cytokines initiates upon 24 hours of incubation. While there are various studies reporting on the induction of proinflammatory cytokine *IL-1 β* , *IL-8* and *TNF α* expression by trichothecenes in various cell systems (Chung et al. 2003; Wong et al. 2001; Wong et al. 1998), there is only one report showing that trichothecenes weakly induce anti-inflammatory *IL-10* cytokine expression in porcine hepatocytes and Kupffer cells (Doll et al. 2009), which is consistent with our findings.

Mycotoxin Composition of the Fungal Fragments

The exact cellular inflammatory response will be correlated to the mycotoxin composition found in the fungal fragment which in turn is highly strain specific making it difficult to generalize across studies. The two fungi used in the present study are known to produce different mycotoxins. *A. versicolor* is best known for producing sterigmatocystin and some strains have been shown to produce trichothecene (Atalla et al. 2003; Atoui et al. 2007; El-Shanawany et al. 2005). Even though most *A. versicolor* strains do not produce trichothecenes, the particular strain used in this study was previously demonstrated to inhibit protein syntheses, which is a known cytotoxic effect caused by macrocyclic trichothecenes, thereby providing indirect evidence for the presence of trichothecenes in the particular strain studied (Dean et al. 2008) and supporting the ELISA measurements reported herein. It has been reported that the submicron fragments of *A. versicolor* are much more immunoreactive than spores which may be due to the mycotoxins found in those fractions (Gorny et al. 2002). These findings are also consistent with the data presented herein. Our results with *S. chartarum* RTI 5802, however, are different than that published by Brasel et al. (2005a). In

Brasel et al.'s study, fungal fragments of *S. chartarum* were collected using a simplified aerosolization chamber and the concentrations of trichothecenes in the fungal fragments were measured using the QuantiTox™ ELISA kit (Brasel et al. 2005a). High concentration of trichothecenes (>100 ppb) were detected in fragments larger than 5 µm (spores) and 1.2-5 µm size but low amount (<1 ppb) were detected in the 0.4-1.2 µm range. The exact reason for the difference between our and Brasel et al.'s results is unclear but may be related to differences in strains or experimental set-ups. In our experimental setting, we used an aerosolization chamber coupled with a cascade impactor lined with aluminum foil to separate and collect particles in five size ranges (<0.25, 0.25-0.5, 0.5-1.0, 1.0-2.5 and >2.5 µm) whereas polycarbonate membrane filters were used to separate the fungal fragments of three size ranges (<0.4, 0.4-1.2, and 1.2-5.0 µm) in the Brasel et al.'s study. In addition, because air was blown into the chamber as opposed to directly onto the fungal surface, it is possible that the larger, and therefore heavier, fragments were never aerosolized in our experiments.

In the literature, there are several reports that mycotoxins or the effects of mycotoxins are not found in fungal fractions larger than 1 micron. Several researchers have reported that spore extracts from *S. chartarum* and *A. versicolor* had weak or no effects on inflammatory cytokine expression in several cell lines (Huttunen et al. 2003; Murtoniemi et al. 2001; Murtoniemi et al. 2005). Gorny et al. (2002) reported that spore fractions of *A. versicolor* had much less immunoreactivity, as carried by immunoreactive molecules such as mycotoxins, than submicron fragments. It should be noted however that these studies did not directly perform mycotoxin concentration measurements but because of their decreased immunoreactivity, their results suggest that mycotoxin levels are decreased in the larger fragment sizes containing spores. The ELISA results presented herein showing that the larger fungal fragments (>2.5 µm, spores) did not contain trichothecenes are consistent with these findings but are, however, different than those presented by Brasel et al. (2005a).

Effects of Mycotoxin on Cell Viability

An intermediary response on cell viability was observed with the fungal extract mixture as compared to the individual strains. This result was surprising as the presence of *S. chartarum* RTI 5802 appeared to attenuate the effect observed when THP-1 cells were incubated in the presence of *A. versicolor* RTI 3843 alone. It should be noted that in our research only half as many cells of each fungal strain were present in the chamber when collecting the fungal extracts for the mixture case (i.e., one agar plate for each type as opposed to two plates for the single fungus exposure), so it is possible that the attenuated effect is simply a result of a dose response or threshold effect of other mycotoxins present on the fragments. It is unlikely to be directly linked to trichothecenes because their concentration is actually higher in the mixture case.

The co-culture of *S. chartarum* RTI 5802 and *A. versicolor* RTI 3843 has previously been demonstrated to cause a synergistic increase in cytotoxicity compared to the sum of response induced by the individual pure cultures (Murtoniemi et al. 2005). There are two possible reasons why our results are different from those results. First, both fungi were co-cultured in a single plate simultaneously whereas in our research fungi were cultured in separate plates but mixed in aerosolization chamber before extraction. Growth of competing microbes on the same culture substrate may influence their tendency to produce toxic secondary metabolites. Secondly, pure spores were extracted and used in the cytotoxicity studies as opposed to fragments in our study. These experimental differences may explain the results and additional research should be carried out to further examine the exact effects of mycotoxins other than trichothecenes produced by fungi.

Mixture Effects of Mycotoxins in Fungal Extracts

Cytokine gene expression was affected even when tricothecene concentrations in fungal extracts were undetectable using ELISA. In addition, despite the differences in tricothecenes concentrations between the recovered fragment sizes (0.25-0.5 μm vs 0.5-1 μm), the resulting effects on cytokine gene expression were indistinguishable from one another suggesting that toxins other than tricothecenes in the extract may elicit an inflammatory response. Even when tricothecene concentrations were below the ELISA detection limits, significant effects were observed on gene expression as well as cellular viability indicating the likely presence of toxins in the 0.5-1.0 μm fungal fragments extracts. It is possible that other mycotoxins which are not detected by the ELISA utilized herein were present in the samples of fungal fragments and caused this effect. There are numerous reports on the synergistic or additive effects of various mycotoxins in crude extracts as compared to individual pure mycotoxins for eliciting cellular responses (Heller et al. 2002; Muller et al. 2003). Heller et al. (2002) reported that crude extracts of *Aspergillus ochraceus* were much more effective at inducing the expression of *TNF α* in THP-1 cells as compared to the pure mycotoxin ocratoxin A at comparable concentrations. Muller et al. (2003) showed that ocratoxin C in a crude extract containing ocratoxin A augmented the immunomodulatory effect of ocratoxin A in THP-1 cells. These results have important implications as fungal contaminations rarely are linked to a single mycotoxin or fungal strain. Our results demonstrate the difficulty in obtaining representative samples which incorporate these factors.

Numerous exposure guidelines have been suggested but there is no general consensus of threshold values which should be used (Gots et al. 2003; Rao et al. 1996). The lack of exposure guidelines is in part due to the difficulty of establishing a unique threshold limit for individuals with different sensitization levels as well as to the lack of data for systemic response to mycotoxin producing fungal exposure. Furthermore, safe exposure limit guidelines regarding the potential health effect based on fungal counts should consider mixture effects when determining possible exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Cytokine expression normalized to GAPDH stimulated by exposure to *S. chartarum* RTI 5802 submicron fragments of varying sizes. Error bars represent $\pm$ one standard deviation. Star (*) indicates significant difference from negative control samples with 95% confidence interval.

Figure 2.

Cytokine expression normalized to GAPDH stimulated by exposure to *A. versicolor* RTI 3843 submicron fragments of varying sizes. Error bars represent $\pm$ one standard deviation. Star (*) indicates significant difference from negative control samples with 95% confidence interval.

Figure 3.

Cytokine expression normalized to GAPDH stimulated by exposure to fungal mixture of *S. chartarum* RTI 5802 and *A. versicolor* RTI 3843 submicron fragments of varying sizes. Error bars represent $\pm$ one standard deviation. Star (*) indicates significant difference from negative control samples with 95% confidence interval.

Figure 4. Effect on THP-1 cell viability. Error bars represent $\pm$ one standard deviation. Pound (#) indicates treatments not significantly different from the 1 ng/mL Roridin A control samples with 95% confidence interval.