

The role of fungal proteinases in pathophysiology of *Stachybotrys chartarum*

Iwona Yike · Thomas Rand · Dorr G. Dearborn

Received: 20 March 2007 / Accepted: 6 June 2007 / Published online: 3 July 2007
© Springer Science+Business Media B.V. 2007

Abstract The adverse health effects of *Stachybotrys chartarum* have often been linked to exposure to the trichothecene mycotoxins. Recent studies have shown that in addition to mycotoxins this fungus is capable of producing and secreting in vivo proteins such as hemolysins and proteinases. Spore extracts obtained from a high trichothecene producing isolate JS 58-17 exhibited a significantly lower proteolytic activity compared to the low trichothecene producer, JS 58-06. Growing isolates on rice or potato dextrose agar results in higher proteolytic activity of the spores compared to those grown on drywall. Proteinases in the spore extracts can hydrolyze gelatin and collagen I and IV. Analysis of zymograms shows the presence of several proteins with proteolytic activity in the spores of *S. chartarum*. Human tracheal epithelial cells exposed to spore extracts produced significantly higher levels of IL-6, IL-8, and TNF- α than control cells. This stimulation of cytokine production was

completely abolished by Pefabloc, a serine protease inhibitor. Neutrophil numbers and proinflammatory cytokine (IL1- β and TNF- α) concentrations were highly elevated in the lungs of 7 day old rat pups exposed intratracheally to 4×10^4 spores/gm body weight compared to control. No significant differences in those inflammatory indices in vivo were noted between the treatments with the high trichothecene producer, isolate JS 58-17 and JS 58-06, which does not produce macrocyclic trichothecenes. Immunohistochemistry revealed reduced collagen IV labeling in spore-induced lung granulomas in rat pups exposed to both isolates. These results suggest that proteinases from *S. chartarum* spores significantly contribute to lung inflammation and injury.

Keywords *Stachybotrys* · Spores · Proteinases · Lung injury

I. Yike · D. G. Dearborn
Mary Ann Swetland Center for Environmental Health,
Case Western Reserve University, Cleveland, OH, USA

I. Yike (✉) · D. G. Dearborn
Department of Environmental Health Sciences, School of
Medicine, Case Western Reserve University, Wood Bldg,
Room WG19; Location code: SOM 494, 2109 Adelbert
Rd., Cleveland, OH 44106, USA
e-mail: ixy@case.edu

T. Rand
St. Mary's University, Halifax, NS, Canada

Introduction

Stachybotrys chartarum is a toxigenic fungus that thrives on water-saturated, cellulose-rich substrates such as straw, hay, cereal grains, wood pulp, paper, and cotton. This mold is capable of producing several classes of mycotoxins including trichothecenes, spirocyclic drimanes, cyclosporine, and atranones [1]. Macrocyclic trichothecenes synthesized by *Stachybotrys chartarum* such as satratoxins H and G and roridin are highly potent inhibitors of protein

synthesis in eukaryotic cells [2, 3]. Trichothecenes exposure through contaminated feed and dust aerosols have been reported to be a cause of stachybotryotoxicoses in domestic animals [4]. In experimental animals, trichothecenes produce biological effects similar to those observed in stachybotryotoxicoses [5].

Stachybotrys chartarum and satratoxins have been detected in the air samples and construction materials from buildings with moisture problems [6–9]. Occupants have suffered from the “sick building” syndrome that includes cough, irritation of the eyes and skin, headache, fatigue, and other respiratory tract symptoms such as wheezing, chronic bronchitis as well as changes in the immune system [10]. An increased incidence of acute pulmonary hemorrhage among infants in Cleveland has been linked to exposure to *S. chartarum* and other molds and has raised public awareness of the risks involved in indoor exposure to molds [11]. Dearborn et al. [12, 13] hypothesized that pulmonary hemorrhage was due to inhalation of trichothecenes associated with aerosolized *S. chartarum* spores, mycelial fragments and substrate dusts. The subsequent inhibition of collagen IV synthesis in developing infant lungs led to capillary wall fragility and increased susceptibility to stress failure and rupture leading to hemorrhage. Studies of the effects of intranasal exposure to the spores of *Stachybotrys chartarum* in mice [14, 15] as well as experiments on young rats [16, 17] and intratracheally instilled infant rats conducted in our laboratory also led to the conclusion that resulting pulmonary inflammation and alveolar hemorrhage was related directly to the trichothecene content of the spores [18]. While those results justified the initial focus on trichothecene mycotoxins, only about 30% of *S. chartarum* isolates belong to the highly toxic, macrocyclic trichothecene producing chemotype [19]. The other chemotype producing atranones and simple trichothecenes is characterized by low toxicity [20]. More recent findings of serious adverse effects of low-trichothecene producing isolates of *S. chartarum* [21–24] indicate that fungal metabolites other than trichothecenes are also involved in eliciting inflammatory responses and lung injury. While some authors refer to yet “obscure agents” [20], we propose that fungal proteins, especially proteinases [25], hemolysin [26] and possibly other enzymes can also lead to the inflammatory effects of *Stachybotrys chartarum*. The goal of this study was to examine a

hypothesis that proteinases from the spores of *S. chartarum* contribute to lung injury both indirectly by activating cellular pathways involved in inflammatory responses and directly by destroying structural proteins such as collagen type IV.

Methods

Fungal spores

Two Cleveland strains of *S. chartarum* JS 58-17 (=ATCC 201211) and JS 58-06 (=ATCC 201863) were grown on drywall (gypsum board) as described before [27] or on potato dextrose agar (PDA). The spores were collected from the surface of the cultures by gently wiping it with a “Q-tip”. Additionally, in some experiments spores obtained from rice cultures kindly provided by Dr. W. Sorenson and Dr. D. Lewis from NIOSH, Morgantown, WV, were used. The purity of spore preparations was assessed microscopically. Trichothecenes were determined by a satratoxin G ELISA [28] and luciferase translation inhibition assay [29].

Animals

Pregnant Sprague-Dawley female rats at 18–19 days of gestation were obtained from Charles River (Wilmington, MA). The rats were housed in microisolators in the CWRU animal facility and fed the standard diet of Teklad 8664 (Harlan) and water ad libitum. Each litter contained between 8 pups and 14 pups. The animal research protocol has been approved by the Case Western University Institutional Animal Care and Use Committee.

Intratracheal instillation of fungal spores

Seven day old newborn rats weighing about 17 g (6 pups per experimental group) were anesthetized with metoxyflurane (Shering-Plough Animal Health Corp., Union, NJ). A transverse skin incision was made and the trachea was exposed by blunt dissection. Fungal spores were suspended in 20 μ l of phosphate buffered saline (PBS) at pH 7.4 containing 0.1% Tween-20 and injected directly into the trachea (4.0×10^4 spores/g body weight for bronchoalveolar lavage (BAL) analysis and 1.0×10^5 for collagen IV

immunohistochemistry) using a 24 G angiocatheter attached to a sterile Hamilton syringe. The incision was closed and treated with New Skin liquid bandage (Medtech Laboratories Inc., Jackson, Wyoming) to facilitate healing and decrease maternal cannibalism. Control animals received PBS with 0.1% Tween-20 intratracheally in the same manner as the animals treated with fungal spores.

Bronchoalveolar lavage

Bronchoalveolar lavage was performed 48 h after exposure. The rats were weighed and the total lung volume (TLV) ($=63 \mu\text{l/g}$ body weight) was calculated for each animal [30]. Following anesthesia and exsanguination, the trachea was cannulated using a 24 G angiocatheter attached to 1 ml tuberculin syringe. The lungs were lavaged three times with sterile PBS volume equal to 75% of TLV. Phenylmethylsulfonyl fluoride (PMSF) at final concentration of 0.1 mM and EDTA (ethylene-diaminetetraacetic acid) at final concentration of 5 mM were added to the bronchoalveolar lavage (BAL) fluid to inhibit proteinases and the BAL fluid was centrifuged for 10 min at 100g. The supernatants were collected and filtered through 0.22 μm syringe filters and stored at -80°C prior to cytokine and urea assays. The pellets were suspended in 1 ml of ice-cold PBS solution and the cell count was determined using hemocytometer. Aliquots of cell suspension were transferred to slides in a cytospin centrifuge (Shandon, Pittsburgh, PA). Following centrifugation the slides were air-dried and stained with the Wright-Giemsa stain. The differential cell count was performed to determine the cell composition of the BAL fluid (200 nucleated cells per slide were counted).

The final concentration of the cells in the BAL fluid was calculated per milliliter of the epithelial lining fluid using the urea dilution factor to correct for the dilution of the BAL fluid. The urea of serum and BAL fluid supernatants was measured by the method of Rennard et al. [31]. The dilution factor for BAL fluid and the volume of epithelial lining fluid (ELF) were calculated for each animal.

Preparation of extracts from the spores of *S. chartarum*

Fungal spores were collected from rice, potato dextrose agar (PDA) and drywall (DW) cultures, suspended in a

solution containing 2 mM Tris/HCl pH 7.4, 100 mM KCl, 1.5% polyvinylpyrrolidone and enumerated in a hemocytometer. Their purity was assessed microscopically to be more than 95%, with the remainder being conidiophores and mycelial fragments.

Spore suspensions containing 5×10^7 spores/ml were then disrupted in a mini-bead beater (Biospec Products, Inc, Bartlesville, OK) with acid washed glass beads (3×20 s interspaced with two 1 min cooling periods on ice). Cell breakage was evaluated microscopically. The suspensions were centrifuged for 20 min at 17,000g and the supernatants collected.

Proteinase activity

Enzymatic activity was measured using 2–5 μg protein of spore extracts and an EnzCheck[®] kit from Molecular Probes (Eugene, Oregon) with fluorescein-labeled gelatin and collagens type I and IV as substrates. The activity was expressed as arbitrary fluorescence units per 1 million spores. Proteinase inhibitors: aprotinin, leupeptin, pepstatin, and Pefabloc SC (AEBSF: 4-(2-aminoethyl) benzenesulfonyl fluoride) were obtained from Roche (Mannheim, Germany). 1, 10-orthophenanthroline was from Molecular Probes (Eugene, Oregon). Tosylphenylalanylchloromethyl ketone, phenylmethylsulfonyl fluoride (PMSF) and ethylenediaminetetraacetic acid (EDTA) were from Sigma (St. Louis, Missouri).

Tracheal epithelial cells

Human tracheal epithelial cells 9/HTEo[−] derived from SV40 transformed human tracheal epithelial cells [32] were kindly provided by Dr. Diane Kube from Dept. of Pediatrics, CWRU. The cells were grown in Dulbecco's minimal Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 2.5 mM L-glutamine in an atmosphere of 95% air–5% CO₂ at 37°C.

The cells were plated at 1.0×10^6 cells per well on Vitrogen coated 24-well plates. Twenty four hours after being plated the cells were switched to serum-free medium for 18 h. The cells were exposed to 1 ml of spore extract diluted in culture media, containing 10 μg of fungal protein/ml, for 2 h at 37°C. The extracts were then removed and replaced with fresh serum-free culture medium. The media were collected after 24 h and analyzed for cytokine content. Epithelial cell

monolayers were lysed with 300 µl of 1× lysis buffer (Promega, Madison, WI), and protein concentration was determined as described below. The results were normalized per 1 mg of lysate protein. The cells from parallel wells were trypsinized and counted using a hemocytometer. Viability of treatment and control cells was assessed by trypan blue exclusion.

Protein assay

Protein content of spore extracts was determined by the method of Bradford using reagents from Biorad (Hercules, California).

Zymography

Spore extracts (2 µg protein/lane) were run on precast 10% gelatin gels obtained from Biorad (Hercules, California). The gels were incubated for 30 min at 37°C in 2% Triton X-100, rinsed three times in 50 mM Tris-HCl pH 8.4 and incubated overnight in 50 mM Tris HCl pH 8.4 containing 0.56 g/l CaCl₂. The gels were then stained with Coomassie brilliant blue and partially destained in ethanol: acetic acid: water (4:1:5).

Cytokine measurements

Human IL-6, IL-8, and TNF-α were measured using the Fluorokine[®] MAP cytokine multiplex kits and the Luminex[®] 100[™] analyzer (Luminex Corporation), detection limit ~5.0 pg/ml. Rat cytokines IL-1β and TNF-α were assayed by ELISA using kits from R&D Systems (Minneapolis, Minnesota) containing affinity purified antibodies to rat cytokines. The limits of detection for both IL1-β and TNF-α were 5.0 pg/ml.

Collagen immunocytochemistry

Immunocytochemical labeling of collagen type IV in the lungs of rats exposed to 100,000 sp/gm BW of *S. chartarum* grown on drywall was performed using a polyclonal goat anti-collagen IV-biotin Fab-fragments as described before [33]. Briefly, 72 h after exposure the rats were exsanguinated under metophane anesthesia, the lungs were fixed using 33% paraformaldehyde in phosphate buffered saline for 48 h, dehydrated through an ascending ethanol series, cleared in xylene,

embedded in paraffin and sectioned at 3 µm thick. Sections were dewaxed and hydrated through a descending ethanol series, washed with 0.05 M Tris-HCl buffer pH 7.6 and placed in boiling 10 mM citrate buffer pH 5.8 for 10 min. Cooled sections were rinsed three times in TBS (Tris buffered saline) buffer pH 7.8, 4 min each. Slides were then placed in 50 ml of 0.5% trypsin solution and incubated at 37°C for 15 min, rinsed twice with TBS solution pH 7.8, 4 min each. Slides were then washed twice in PBS (phosphate buffered saline) solution pH 7.6, 4 min each and blocked in 10% lamb serum at room temperature for 30 min. The slides were then incubated with polyclonal goat anti-collagen IV-BIOT Fab fragments (Sigma, St. Louis, MO) diluted 1:40 in 0.01 M PBS (pH 7.8) for 2 h at RT in a humid chamber. Control slides were exposed to PBS only. The slides were then rinsed 2 × 4 min in PBS followed by 2 × 4 min Tris HCl pH 7.6. After rinsing slides were incubated with anti-rabbit-BIOT diluted 1:1,000 at RT for 1 h. After incubation they were rinsed 3× with 0.01 M Tris HCL pH 7.6 and exposed to anti-biotin alkaline phosphatase conjugated Fab fragments (Roche) diluted 1:30,000 for 1 h at RT. After rinsing 3 × 4 min each with Tris-HCl buffer pH 8.0 followed by Tris HCl buffer pH 9.5 for 10 min. To develop a color reaction the slides were incubated with a solution of NBT/BCIP (Sigma, St.Louis, MO) at 37°C for 3.5 h, rinsed with water, dehydrated through an ascending ethanol series, cleared in xylene and mounted using Permount.

Statistical analysis

The results were analyzed using ANOVA and SigmaStat 2.03 software from Jandel Scientific (San Rafael, CA). The differences between experimental groups and treatments were considered statistically significant when $P \leq 0.05$. All data are expressed as mean ± SEM.

Results

Proteolytic activity and trichothecene toxicity of spore extracts

As shown in Fig. 1a, proteolytic activity of spore extracts from the low trichothecene producing isolate JS 58-06 is more than 2-fold higher than from the

high trichothecene producing isolate JS 58-17. The activity of each isolate was significantly increased ($P < 0.005$) when it was grown on rice or potato dextrose agar (PDA) rather than on drywall (DW)—nearly 5-fold higher for JS58-06 and about 3.5-fold higher for JS58-17. Proteinases from both isolates hydrolyzed gelatin faster than collagen I which in turn was hydrolyzed faster than collagen IV. Trichothecene toxicity of JS 58-17 was nearly 10^4 -fold higher than JS58-06 and was not affected by the growth conditions (Fig. 1b).

When the spore extracts were separated on gelatin zymogram gels, several major protein bands with proteolytic activity were obtained, with an apparent molecular weight of 200, 180, 140, 130, 94, and 30, 26 and 22 kDa (Fig. 2). Differences in the profile and

intensity of several bands between the two isolates of *S. chartarum* were also apparent and were affected by the growth media. Similar to the results shown in Fig. 1, zymography demonstrated that JS58-06 isolate had a higher proteolytic activity than JS58-17 (Fig. 2a–c and d–f). For both isolates, the level of proteolytic activity of the spore extracts obtained from rice and PDA cultures was higher than that of the spore extracts obtained from drywall.

When JS56-06 was grown on rice, the most prominent proteinases present in the spores were those with a molecular weight of 94, 30, and 26 kDa (Fig. 2, lane a). Growing the same isolate on PDA led to the increased activity of proteolytic bands with a higher molecular weight (94–200 kDa—Fig. 2, lane b) and the presence of a single band at 30 kDa. The spores grown on drywall had a lower proteolytic activity found mostly in the 30 kDa band while the 94 kDa band was missing (Fig. 2, lane c).

The spores of JS58-17 grown on rice had three major proteinases of 130, 30, and 26 kDa (Fig. 2, lane d), while in those grown on PDA the major band was that of 22 kDa and the minor bands of 94–200 kDa and 54 kDa were also detected (Fig. 2, lane e). Greatly reduced proteolytic activity and only two

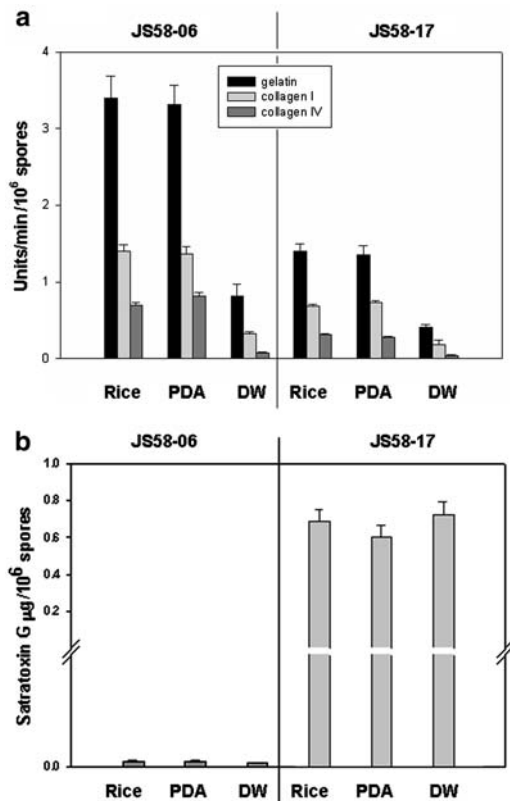


Fig. 1 (a) Proteolytic activity of spore extracts from two isolates of *S. chartarum*, JS 58-06 and JS58-17 grown on rice, potato dextrose agar (PDA), and drywall (DW). Proteolytic activity was measured using three different protein substrates: gelatin, collagen I, and collagen IV. (b) Satratoxin G content of the same spore extracts measured by ELISA. Values are mean $\pm$ SEM, $n = 3$

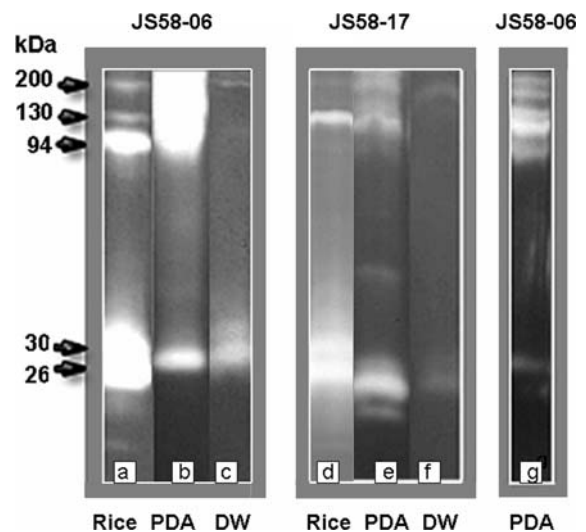


Fig. 2 Gelatin zymography of spore extracts (2 µg protein/lane) from two isolates of *S. chartarum* JS58-06 and JS58-17 grown on three different media: (a) JS58-06 on rice, (b) JS58-06 on potato dextrose agar (PDA), (c) JS58-06 on drywall (DW), (d) JS58-17 on rice, (e) JS58-17 on PDA, (f) JS58-17 on DW, (g) same as “b” but the protein content was reduced five times to clearly visualize the bands

bands of 180 and 22 kDa were seen in the extracts from the spores of this isolate grown on drywall (Fig. 2, lane f).

This pattern of proteolytic band distribution was somewhat variable between different experiments and changed with the age of the cultures.

Inhibitors of proteolytic activity

Inhibition of proteolytic activity of spore extracts was studied using the more abundant source of spore proteinases, JS58-06 from PDA cultures. Proteolytic activity of spore extracts from *S. chartarum* was significantly inhibited only by class-specific inhibitors of serine proteinases. As shown in Table 1, PMSF (phenylmethylsulfonyl fluoride—2 mM) and Pefabloc (4 and 8 mM) strongly inhibited the proteolytic activity of spore extracts while a lower concentration of TPCK (tosylphenylalanyl-chloromethyl ketone—0.5 mM) was less effective. Aprotinin was not effective at concentrations 0.75 μ M and above. 1, 10-orthophenanthroline had a minimal effect and there was no change in the enzymatic activity when leupeptin, pepstatin and EDTA were used.

In vitro effects of spore extracts on tracheal epithelial cells

When tracheal epithelial cells 9/HTEo[−] were exposed to extracts from JS 58-06 spores collected from PDA cultures, concentrations of secreted IL-8 and IL-6 increased more than 10-fold, while TNF- α levels were elevated about 3-fold after 24 h (Fig. 3).

Table 1 Inhibition of proteases from the spores of *Stachybotrys chartarum*

Inhibitor	Concentration	Residual activity (%)
Aprotinin	0.75 μ M	97
Leupeptin	10.00 μ M	100
Pepstatin	10.00 μ M	100
EDTA	2.50 mM	100
1,10-orthophenanthroline	2.50 mM	96
PMSF	2.00 mM	25
Pefabloc	4.00 mM	71
	8.00 mM	49
TPCK	0.50 mM	83

Addition of serine proteinase inhibitor (2 mM Pefabloc) almost completely abolished these increases. Pefabloc alone slightly inhibited the release of IL-8 and IL-6 below the control level but did not affect base line secretion of TNF- α . Viability of the cells exposed to the spore extract at the end of treatment was 89%.

The extracts derived from the same number of spores from the highly toxic isolate JS58-17 caused a rapid loss of viability and detachment of cells from the surface of culture plates making collection of data parallel to the isolate JS58-06 impossible.

In vivo effects of two isolates of *S. chartarum*

The analysis of bronchoalveolar lavage fluid (BALF) shows that intratracheal exposure of 7 day old infant rats to the spores from two isolates of *S. chartarum* led to severe lung inflammation. The neutrophils and proinflammatory cytokines, TNF- α and IL-1 β were among the most sensitive indicators of fungal exposure (Fig. 4).

At 4×10^4 spores/gm body weight, the effect of both *S. chartarum* isolates on neutrophil abundance was similar when comparing both isolates grown on the same medium. However, the numbers of neutrophils in animals exposed to the spores grown on PDA were much higher ($P = 0.01$) compared to those grown on drywall. Additionally, BALF protein levels were significantly elevated ($P > 0.02$) in animals exposed to PDA grown JS58-17 compared to those exposed to drywall grown JS58-17 and both PDA and drywall grown JS 58-06.

Proinflammatory cytokines remained below the limit of detection in the bronchoalveolar fluid obtained from animals treated with PBS-0.1% Tween-20. Spores from both isolates obtained from PDA caused significantly increased ($P = 0.01$ for 58-17, and $P = 0.048$ for 58-06) IL-1 β concentrations in the BAL fluid compared to the spores derived from drywall. Very small differences ($P > 0.05$) were noted between the effects of spores from the two isolates grown on the same medium; they were slightly higher for JS58-06. The levels of TNF- α follow the same pattern as neutrophils and IL-1 β ; they were significantly higher ($P \leq 0.04$) in the animals exposed to the spores collected from PDA cultures compared to those from drywall.

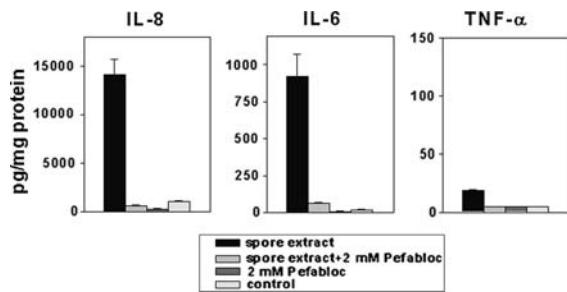


Fig. 3 Proinflammatory cytokine secretion by human tracheal epithelial cells exposed to the extract from the spores of *S. chartarum* JS58-06 grown on PDA. Values are mean $\pm$ SEM, $n = 3$. All values were significantly different from controls ($P < 0.05$)

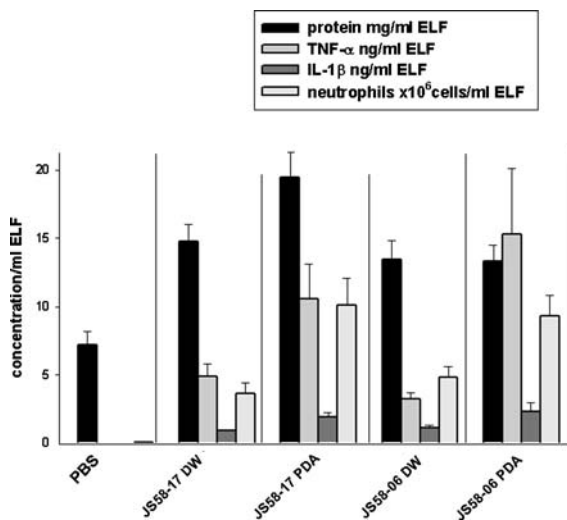


Fig. 4 In vivo effects of intratracheal exposure of infant rats to the spores of two isolates of *S. chartarum* grown on drywall (DW) and potato dextrose agar (PDA). Values are mean $\pm$ SEM, $n = 6$. All values were significantly different from controls ($P < 0.05$)

Collagen type IV in vivo

Control lungs from 7 day old infant rats showed collagen IV labeling indicated by deeply basophilic staining of alveolar walls (Fig. 5a). Immunohistochemistry also revealed collagen IV labeling in alveolar walls thickened as a consequence of inflammation due to the exposure to 1×10^5 spores/gm BW of *S. chartarum* grown on drywall (Fig. 5b), and at the periphery of granulomas surrounding *S. chartarum* spores (Fig. 5c, d). However, reduced labeling was noted in granulomas immediately surrounding spores of both *S. chartarum* isolates, JS58-06 (Fig. 5c) and

JS58-17 (Fig. 5d). There was no apparent difference in collagen IV labeling in JS58-06 or JS58-17 spore-induced lung granulomas.

Discussion

Fungal proteinases have been implicated in allergic responses to molds and a number of such enzymes have been identified as major fungal antigens [34]. They have also been considered as virulence factors in infectious mycoses [35]. In contrast to many bacterial proteinases, their role in pulmonary injury and the mechanism of inflammatory responses have been studied only recently [36].

We have shown the presence of multiple proteinases in the spores from two different isolates representing two distinct chemotypes of *S. chartarum*, one producing macrocyclic trichothecene toxins (JS58-17), and the other producing atranones (JS58-06) [20]. Our results clearly demonstrate that these two isolates differ significantly in proteolytic activity with the less toxic isolate having higher activity. The same isolate, JS 58-06 was also shown to have more than 6-fold higher content of stachylysin compared to JS-5817 [23]. This finding together with studies by Leino et al. [21] and Flemming et al. [22] sheds new light on the clinical significance of less-toxic isolates that may constitute as much as two thirds of all the *S. chartarum* isolates found in the indoor environment [19]. Our results also show that proteolytic activity and or/protein production, but not trichothecene content of the spores (supporting earlier work by Yike and Dearborn, ref. 23), is influenced by culture medium composition. Growth of *S. chartarum* on rice and PDA resulted in higher proteolytic activity than on drywall. This is not altogether surprising since it is well-known that the medium composition affects fungal metabolism and metabolite production and that richer media induce protein secretion [37]. Nevertheless, this knowledge should figure in on in vivo studies evaluating effects of fungal spores and their constituents on lung pathophysiology. Fungi used in such studies should be grown on media that approximate the composition of ones supporting fungal growth indoors (e.g., drywall) in order to enhance inter-study comparisons.

Proteinase inhibitor work revealed that serine proteinases constitute a large fraction of proteolytic

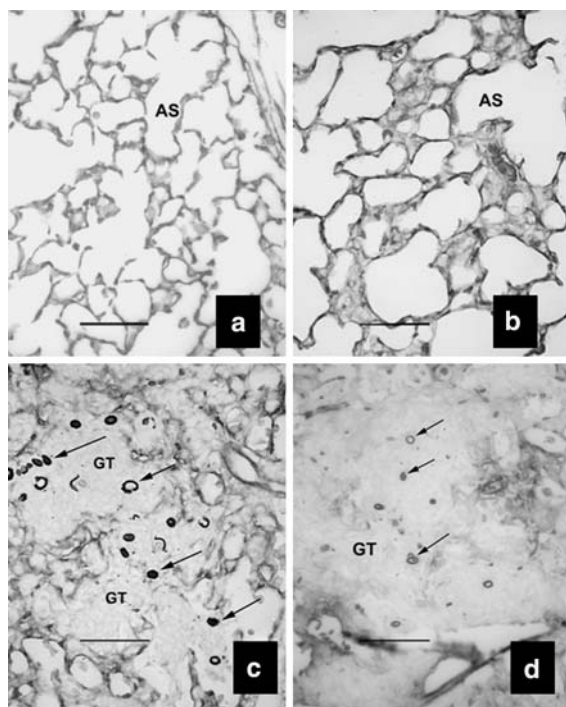


Fig. 5 Collagen IV labeled lungs of infant rats exposed to the spores of two isolates of *S. chartarum* grown on drywall. (a) Collagen IV labeled normal lung tissue from a PBS treated animal. (b) Collagen IV labeled hyperplastic lung tissue from a *S. chartarum* 58-06 treated rat. (c) Collagen IV labeled rat lung granuloma tissue (GT) from a *S. chartarum* 58-06 spore (arrows) exposed animal. Note the apparent lack of labeling in granuloma surrounding spores. (d) Collagen IV labeled rat lung granuloma tissue (GT) from a *S. chartarum* 58-17 spore (arrows) exposed animal. Note the apparent lack of labeling in granuloma surrounding spores. Bar scale: a and b—50 μ m; c and d—20 μ m

activity present in and/or secreted by the spores of *S. chartarum*. The only proteinase from *S. chartarum* that has been characterized to date is stachyrase A [38]. It was isolated from liquid culture media of another Cleveland isolate of *S. chartarum*, JS 51-06, and it is not clear whether it is produced by mycelia or spores, or both. It is a serine protease with a broad substrate specificity that is capable of hydrolyzing several collagens (type I, VI, and X), all four pulmonary proteinase inhibitors (α_2 -macroglobulin, α_1 -proteinase inhibitor, α_1 -antichymotrypsin, secretory leukocyte protease inhibitor = SLPI), and several neuropeptides (substance P, bradykinin, neurotensin, and angiotensin I and II). The fact that the two major proteolytic bands in the spore extracts (26 and 30 kDa) are close in size to that of stachyrase A suggests that at least one of them

may correspond to that enzyme. However, more studies are needed. Further characterization of proteinases from the spores of *S. chartarum* would require isolation of a number of enzymes. Isolating those that may be spore-specific by conventional biochemical approaches would require using large amounts of spores which may not be practical.

Our attention was focused on *S. chartarum* enzymes produced by the spores which until recently have been considered the main respirable fungal particles [9, 39]. Although many proteins are produced both by growing mycelia and by the spores, some of them may be spore specific [40]. Similarly to stachyrase A, proteinases from the spores were able to digest collagen I and also collagen IV. The lack of inhibition by orthophenanthroline and EDTA (specific collagenase inhibitors) indicates that these collagenolytic proteinases are not true collagenases but rather, similar to stachyrase, serine proteinases with collagenolytic activity.

Earlier studies from our laboratory [18, 23, 41] as well as those by other workers [14–17, 22, 33] have clearly shown that spore exposures precipitate inflammatory responses. These responses can be modulated by a variety of factors including toxin type and toxin content, allergen content and other spore characteristics.

The effects of spore extracts on the tracheal cells in vitro point to the involvement of fungal proteinases in the induction of the inflammatory process. The proteinase effects can be differentiated from toxin effects because when serine proteinase inhibitors were added to the medium, the production and/or secretion of proinflammatory cytokines IL-6, IL-8 and TNF- α in human tracheal cells was practically abolished. This indicates that proteinase activity is needed to elicit inflammatory responses, possibly through proteinase activated receptors (PARs), as suggested by Vliagoftis et al. [42]. While the results presented here agree with the earlier findings of Kauffman et al. [36] suggesting that fungal proteinases were involved in the induction of cytokine secretion, the role of proteinases in the pathophysiology of *S. chartarum* has not been discussed before.

Initial studies of the effects of *S. chartarum* on the lungs of laboratory animals [14–18, 43] suggested that trichothecene toxins from the spores were primarily responsible for inflammatory response and subsequent injury. These conclusions were based on the observation that spores extracted with ethanol or methanol did

not elicit inflammatory responses in the lungs of exposed animals. However, in addition to removing mycotoxins, alcohol treatment is likely to extract and/or denature many of the fungal proteins and eliminate active secretion of different compounds. Early observation by Nikulin et al. [14] also suggested that the spores from an isolate that does not produce macrocyclic trichothecenes had very mild pulmonary effects. In contrast, more recent studies of Leino et al. [21], Flemming et al. [22], Hudson et al. [24] and those from our laboratory [23] showed that even the isolates of *S. chartarum* that do not produce macrocyclic trichothecenes are capable of eliciting strong inflammatory responses in exposed animals. Nielsen et al. [20] proposed that trichothecene toxins are responsible for the acute toxic effects of the spore inhalation while other fungal agents may be responsible for the induction of inflammatory responses. We postulated that fungal proteins and enzymes including proteinases and hemolysin constitute such agents [23]. Our recent studies comparing the effects of intact, ethanol-extracted, and autoclaved spores from *S. chartarum* isolate JS58-17 show that the viable spores have the strongest effects on all of the inflammatory indices studied [41]. In those studies, autoclaved spores had somewhat milder effects and the effects of ethanol-extracted spores were even weaker. Since proteins appeared to be released from the spores in the lung more slowly than trichothecenes, we expected that the acute toxic effects such as the protein leakage will precede the inflammatory responses. While the BALf protein was already elevated after 3 h and most of the inflammatory effects such as the elevated levels of neutrophils, erythrocytes and proinflammatory cytokines peaked at around 24 h post-exposure, there was no clear separation in time between the acute toxin effects and the later action of fungal proteins. Rather, it appears that there might be some synergistic effects between trichothecenes and fungal proteins. In vivo effects of the spores of *S. chartarum* derived from two isolates grown on two different media described in this report show that increases in the levels of neutrophils and cytokines correspond to the increased proteolytic activity of the spore preparations (see Fig. 1 and 4). The inflammatory effects of highly toxigenic JS 58-17 in rat pups differ only slightly from those of JS 58-06 but there are large, statistically significant differences between the effects of the same isolate grown under different conditions. Those differences cannot be

attributed to different trichothecene toxicity because growth media did not affect the levels of trichothecenes produced by the spores. However, in addition to trichothecenes, proteinases, and hemolysins, other fungal metabolites may be involved. Atranones produced by the isolates of *S. chartarum* that do not produce macrocyclic trichothecenes are likely to be among factors contributing to lung injury. In contrast to the earlier report of Nielsen et al. [20] showing their low toxicity and lack of the ability to elicit inflammatory responses, Rand et al. [44] recently demonstrated that pure atranones cause inflammation in an in vivo mouse model of lung disease.

In addition to the effects of fungal proteinases on lung inflammation, they may also directly affect the turnover and deposition of collagen IV. Our initial hypothesis explaining the link between the exposure to *S. chartarum* and pulmonary hemorrhage assumed that inhibition of the synthesis of type IV collagen by trichothecenes in the growing lung may be the primary mechanism responsible for pulmonary hemorrhage. We postulated that destruction of type IV collagen may lead to fragile capillaries that can in turn result in overt pulmonary hemorrhage as observed in infants exposed to *S. chartarum* through inhalation of indoor air [11, 13]. A subsequent study by Rand et al. [33] describing changes in collagen IV expression in mouse lungs exposed to the spores of a high macrocyclic trichothecene producer, *S. chartarum* isolate JS-58-17 concluded that trichothecene exposure resulting in concurrent inhibition of collagen synthesis may not fully explain the observed effects. Based on the studies of Vesper et al. [26] and Yike et al. [25], the authors suggested that toxins other than trichothecenes and fungal proteins such as stachylysin and proteinases may modulate the pathways responsible for collagen IV synthesis and deposition in the lung. The lack of significant differences in the staining for this important component of the basement membrane in the lungs of rats exposed to the high trichothecene producing isolate JS 58-17 and JS 58-06, that does not produce macrocyclic trichothecenes, agrees with such interpretation. In case of exposure to JS58-17, the reduced levels of collagen IV may result primarily from the inhibition of protein synthesis by trichothecenes, while more active proteinases from JS58-06 may be playing a larger role in collagen degradation.

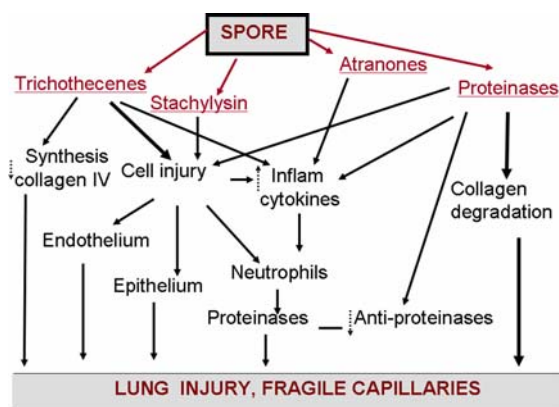


Fig. 6 Possible mechanisms of *S. chartarum* induced lung injury

Figure 6 illustrates some of the pathways that may be involved in lung injury and may lead to pulmonary hemorrhage resulting from exposure to the spores of *S. chartarum*, including the effects of trichothecenes, atranones, proteinases, and hemolysin(s) and their possible synergistic interactions.

Although further studies are needed to fully elucidate those mechanisms, the two lines of evidence regarding the involvement of fungal proteinases obtained from both in vivo and in vitro studies presented in this report support the general scheme.

Acknowledgments This work was supported by NIEHS grant ES09742. The authors thank Jody Gower and Carrie McCaughy for excellent technical assistance.

References

- Miller JD, Rand TG, Jarvis BB. *Stachybotrys chartarum*: a really malicious mold. *Med Mycol* 2003;41:271–91.
- Ueno Y. Mode of action of trichothecenes. *Ann Nutr Aliment* 1977;31:885–900.
- Karunasena E, Cooley JD, Straus D, Straus DC. Protein translation inhibition by *Stachybotrys chartarum* conidia with and without the mycotoxin containing polysaccharide matrix. *Mycopathologia* 2004;158:87–97.
- Harrach B, Bata A, Bajmocy E, Benko M. Isolation of satratoxins from the bedding straw of a sheep flock with fatal stachybotryotoxicosis. *Appl Environ Microbiol* 1983;45:1419–22.
- Pang VF, Lambert RL, Felsburg PJ, Beasley VR, Buck WB, Haschek WM. Experimental T-2 toxicosis in swine following inhalation exposure: effects on pulmonary and systemic immunity and morphologic changes. *Toxicol Pathol* 1987;15:308–19.
- Croft WA, Jarvis BB, Yatawara CS. Airborne outbreak of trichothecene toxicosis. *Atm Environ* 1986;20:549–52.
- Andersson MA, Nikulin M, Koljal U, Andersson MC, Rainey F, Reijula K, et al. Bacteria, molds, and toxins in water-damaged building materials. *Appl Environ Microbiol* 1997;63:387–93.
- Jarvis BB, Sorenson WG, Hintikka E-L, Nikulin M, Zhou Y, Yang J, et al. Study of toxin production by isolates of *Stachybotrys chartarum* and *Memnoniella echinata* isolated during a study of pulmonary hemosiderosis in infants. *Appl Environ Microbiol* 1998;64:3620–5.
- Brasel TL, Douglas DR, Wilson SC, Straus DC. Detection of airborne *Stachybotrys chartarum* macrocyclic trichothecene mycotoxins on particulates smaller than conidia. *Appl Environ Microbiol* 2005;71:114–22.
- Johanning E, Biagini R, Hull D, Morey P, Jarvis B, Landsbergis P. Health and immunology study following exposure to toxigenic fungi (*Stachybotrys chartarum*) in a water-damaged office environment. *Int Arch Occup Environ Health* 1996;68:207–18.
- Etzel RA, Montana E, Sorenson WG, Kullman J, Allan TM, Dearborn DG. Acute pulmonary hemorrhage in infants associated with exposure to *Stachybotrys atra* and other fungi. *Arch Pediatr Adolesc Med* 1998;152:757–62.
- Dearborn DG, Yike I, Miller MJ, Etzel RA. An overview of the investigations into pulmonary hemorrhage in infants in Cleveland, Ohio. *Environ Health Perspect* 1999;107:495–9.
- Dearborn DG, Dahms BB, Allan TM, Sorenson WG, Montana E, Etzel RA. Clinical profile of 30 infants with acute pulmonary hemorrhage in Cleveland. *Pediatrics* 2003;110:627–37.
- Nikulin M, Reijula K, Jarvis BB, Hintikka E-L. Experimental lung mycotoxicosis in mice induced by *Stachybotrys atra*. *Int J Exp Pathol* 1996;77:213–8.
- Nikulin M, Reijula K, Jarvis BB, Veijalainen P, Hintikka E-L. Effects of intranasal exposure to spores of *Stachybotrys atra* in mice. *Fund Appl Toxicol* 1997;35:182–8.
- Rao CY, Brain JD, Burge HA. Reduction of pulmonary toxicity of *Stachybotrys chartarum* spores by methanol extraction of mycotoxins. *Appl Environ Microbiol* 2000;66:2817–21.
- Rao CY, Burge HA, Brain JD. The time course of responses to intratracheally instilled toxic *Stachybotrys chartarum* spores in rats. *Mycopathologia* 2000;149:27–34.
- Yike I, Miller MJ, Tomashefski J, Walenga R, Dearborn D. Infant rat model of *Stachybotrys chartarum* induced mycotoxicosis. *Mycopathologia* 2001;154:139–52.
- Jarvis BB. Chemistry and toxicology of molds isolated from water damaged buildings. In: DeVries JW, Trucksess MW, Jackson SL, editors. *Mycotoxins and food safety*. Kluwer Academic/Plenum Publishers; 2002. p. 43–52.
- Nielsen KF, Huttunen K, Hyvaerinen A, Andersen B, Jarvis BB, Hirvonen M-R. Metabolite profiles of *Stachybotrys* isolates from water-damaged buildings and their induction of inflammatory mediators and cytotoxicity in macrophages. *Mycopathologia* 2001;154:201–5.
- Leino M, Makela M, Reijula K, Haahtela T, Mussalo-Rauhamaa H, Tuomi T, et al. Intranasal exposure to a damp building mould, *Stachybotrys chartarum*, induces lung inflammation in mice by satratoxin-independent mechanisms. *Clin Exp Allergy* 2003;33:1603–10.

22. Flemming J, Hudson B, Rand TG. Comparison of inflammatory and cytotoxic lung responses in mice after intra-tracheal exposure to spores of two different *Stachybotrys chartarum* strains. *Toxicol Sci* 2004;78:267–76.
23. Yike I, Dearborn DG. Pulmonary effects of *Stachybotrys chartarum* in animal studies. *Adv Appl Microbiol* 2004;55:241–73.
24. Hudson B, Flemming J, Sun G, Rand TG. Comparison of immunomodulator mRNA and protein expression in the lungs of *Stachybotrys chartarum* spore-exposed mice. *J Toxicol Environ Health* 2005;68:1321–35.
25. Yike I, Rand T, Dearborn D. Proteinases from the spores of *S. chartarum*. *Am J Respir Crit Care Med* 2002;165:A 537.
26. Vesper SJ, Magnuson S, Dearborn D, Yike I, Haugland RA. Initial characterization of the hemolysin from *Stachybotrys chartarum*. *Infect Immun* 2001;69:912–6.
27. Yike I, Vesper S, Tomashefski J, Dearborn D. Germination, viability and clearance of *Stachybotrys chartarum* in the lungs of infant rats. *Mycopathologia* 2003;56:67–75.
28. Chung Y-J, Jarvis BB, Tal H, Pestka JJ. Immunochemical assay for satratoxin G and other macrocyclic trichothecenes associated with *Stachybotrys chartarum*. *Toxicol Mech Methods* 2003;13:1–7.
29. Yike I, Allan T, Sorenson W, Dearborn D. Highly sensitive protein translation assay for trichothecene toxicity in airborne particulates: comparison with cytotoxicity assays. *Appl Environ Microbiol* 1999;65:88–94.
30. Sahebhami H. Aging of the normal lung. In: Parent RA, editor. *Comparative biology of the normal lung*. Boca Raton: CRC Press; 1992. p. 351–66.
31. Rennard SI, Basset G, Lecossier D, O'Donnell KM, Pinkston P, Martin PG, et al. Estimation of volume of epithelial lining fluid recovered by lavage using urea as a marker of dilution. *J Appl Physiol* 1986;60:532–8.
32. Gruenert DC, Basbaum CB, Welsh MJ, Li M, Finkbeiner WE, Nadel JA. Characterization of human tracheal epithelial cells transformed by an origin-defective simian virus 40. *Proc Natl Acad Sci* 1988;85:559951–5.
33. Rand TG, White K, Logan A, Gregory L. Histological, immunohistochemical and morphometric changes in lung tissue in juvenile mice experimentally exposed to *Stachybotrys chartarum* spores. *Mycopathologia* 2003;156:119–31.
34. Stewart GA, Thompson PJ. The biochemistry of common aeroallergens. *Clin Exp Allergy* 1996;26:1020–44.
35. Millon L, Drobacheff C, Piarroux R, Monod M, Reboux G, Laurent R, et al. Longitudinal study of anti-*Candida albicans* mucosal immunity against aspartic proteinases in HIV-infected patients. *J Acquir Immune Defic Syndr* 2001;26:137–44.
36. Kauffman HF, Tomee JFC, van de Riet MA, Timmerman AJB, Borger P. Proteinase-dependent activation of epithelial cells by fungal allergens leads to morphologic changes and cytokine production. *J Allergy Clin Immunol* 2000;105:1185–93.
37. Pavlukova EB, Belozersky MA, Dunaevsky YE. Extracellular proteolytic enzymes of filamentous fungi. *Biochemistry (Russian)* 1998;63:1059–89.
38. Kordula T, Banbula A, Macomson J, Travis J. Isolation and properties of Stachyrase A, a chymotrypsin-like serine proteinase from *Stachybotrys chartarum*. *Infect Immun* 2002;70:419–21.
39. Madsen AM, Wilkins K, Poulsen OM. Micro-particles from fungi. In: Johanning E, editor. *Fungi, bacteria, mycotoxins and human health*. Albany, New York: Fungal Research Group Foundation, Inc.; 2005. p. 276–81.
40. Schmechel D, Simpson JP, Beezhold D, Lewis DM. The development of species-specific immunodiagnostics for *Stachybotrys chartarum*: the role of cross-reactivity. *J Immunol Methods* 2006;309:150–9.
41. Yike I, Rand T, Dearborn DG. Acute inflammatory responses to *Stachybotrys chartarum* in the lungs of infant rats: time course and possible mechanisms. *Toxicol Sci* 2005;84:408–17.
42. Vliagoftis H, Schwingshackl A, Milne CD, Duszyk M, Hollenberg MD, Wallace JL, et al. Proteinase-activated receptor-2-mediated matrix metalloproteinase-9 release from airway epithelial cells. *J Allergy Clin Immunol* 2000;106:527–45.
43. 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. *Nat Toxins* 1998;6:27–33.
44. Rand TG, Flemming J, Miller JD, Womiloju TO. Comparison of inflammatory responses in mouse lungs exposed to atranones A and C from *Stachybotrys chartarum*. *J Toxicol Environ Health Part A*. 2006;69:1239–51.