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REVIEW

Stachybotrys chartarum, Trichothecene Mycotoxins, and Damp Building–Related Illness: New Insights into a Public Health Enigma

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Damp building–related illnesses (DBRI) include a myriad of respiratory, immunologic, and neurologic symptoms that are sometimes etiologically linked to aberrant indoor growth of the toxic black mold, *Stachybotrys chartarum*. Although supportive evidence for such linkages is limited, there are exciting new findings about this enigmatic organism relative to its environmental dissemination, novel bioactive components, unique cellular targets, and molecular mechanisms of action which provide insight into the *S. chartarum's* potential to evoke allergic sensitization, inflammation, and cytotoxicity in the upper and lower respiratory tracts. Macrocyclic trichothecene mycotoxins, produced by one chemotype of this fungus, are potent translational inhibitors and stress kinase activators that appear to be a critical underlying cause for a number of adverse effects. Notably, these toxins form covalent protein adducts *in vitro* and *in vivo* and, furthermore, cause neurotoxicity and inflammation in the nose and brain of the mouse. A second *S. chartarum* chemotype has recently been shown to produce atranones—mycotoxins that can induce pulmonary inflammation. Other biologically active products of this fungus that might contribute to pathophysiologic effects include proteinases, hemolysins, β-glucan, and spirocyclic drimanes. Solving the enigma of whether *Stachybotrys* inhalation indeed contributes to DBRI will require studies of the pathophysiologic effects of low dose chronic exposure to well-characterized, standardized preparations of *S. chartarum* spores and mycelial fragments, and, coexposures with other environmental cofactors. Such studies must be linked to improved assessments of human exposure to this fungus and its bioactive constituents in indoor air using both state-of-the-art sampling/analytical methods and relevant biomarkers.

Key Words: **lung; respiratory system; nervous system; neurotoxicology; nose; respiratory toxicology; natural products; agents; inflammation; immunotoxicology.**

DAMP BUILDING–RELATED ILLNESSES AND THE *STACHYBOTRYS CHARTARUM* ENIGMA

Adverse human health effects have been attributed to damp indoor air environments resulting from extreme condensation, failure of water-use devices, or building envelope breach during flooding (Institute of Medicine, 2004). Damp building–related illnesses (DBRI) are frequently linked to aberrant indoor growth of microbes, most notably fungi, and include a myriad of respiratory, immunologic, and neurologic symptoms (Andersson *et al*., 1997; Nielsen 2003). An Institute of Medicine (IOM) expert committee concluded that although there is sufficient scientific evidence for an association between the exposure to moldy, damp indoor environments and upper respiratory tract symptoms as well as some lower respiratory tract symptoms, supportive data are insufficient for other specific health conditions such as airflow obstruction, mucous membrane irritation, chronic obstructive pulmonary disease, pulmonary hemorrhage, neurologic effects, and cancer (Institute of Medicine, 2004).

The fungus *Stachybotrys chartarum*, a saprophyte that grows on wet cellulose–containing building materials including wallboard, ceiling tiles, and cardboard (Andersson *et al*., 1997; Boutin-Forzano *et al*., 2004; Tuomi *et al*., 1998, 2000) is often found in low concentrations among the mycoflora identified in water-damaged buildings (Kuhn *et al*., 2005; Shelton *et al*., 2002) as well as representative U.S. housing (Vesper *et al*., 2007). The capacity of *Stachybotrys* to produce biologically potent mycotoxins and its previous association with animal mycotoxicoses has resulted in this fungus being colloquially referred to as "toxic black mold." Two toxic "chemotypes" of *S. chartarum* exist. One chemotype elaborates highly toxic macrocyclic trichothecene mycotoxins, whereas a second chemotype produces less toxic atranones and simple trichothecenes but not macrocyclic trichothecenes (Andersen, 2002).

Case reports have attributed chronic indoor *S. chartarum* exposures to debilitating respiratory symptoms (Croft *et al*.,

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1986; Hodgson *et al*., 1998; Johanning, 1995) as well as nonrespiratory effects suggestive of immune dysfunction (Johanning *et al*., 1996) and cognitive impairment (Gordon, 1999). Based on these and other reports, *S. chartarum* and its products or components have been postulated to etiologically contribute to DBRI (Dearborn *et al*., 1999, 2002; Fung *et al*., 1998; Hossain *et al*., 2004; Jarvis *et al*., 1986, 1998; Kilburn, 2004). Incidences of indoor *Stachybotrys* contamination often generate huge litigation and remediation costs, receive intense media coverage, and have evoked extensive scientific and public controversy. The chief arguments against *S. chartarum* causing adverse human effects are based on the predication that the quantities of this fungus' conidiospores or its toxins required for eliciting systemic adverse effects in animal models exceed levels encountered to date in even the most highly contaminated indoor environments (Chapman *et al*., 2003; Hardin *et al*., 2003; Hossain *et al*., 2004; Lai, 2006). This view does not account for the local tissue injury occurring in the immediate deposition site of the inhaled fungal particles containing high concentrations (millimolar) of deleterious toxins. Nevertheless, it still remains that there are formidable challenges to assessing the inhalation risks of even a single anthropogenic toxicant such as formaldehyde (McGregor *et al*., 2006) much less a complex agent like *S. chartarum*. Unraveling the enigma of *S. chartarum*–mediated pathophysiology and potential etiological links to DBRI against the background of a complex microbial community represents a most daunting task. Despite widespread public attention, until recently, there has been relatively little mechanistic research to address this issue.

Does *S. chartarum* have unique properties compared with other indoor molds that enable it to negatively affect human health and, ultimately, contribute to DBRI? Possible mechanisms by which *Stachybotrys* could adversely impact health include (1) infection, (2) allergy, (3) mycotoxicosis, and/or (4) inflammation. Although the mold's potential to evoke such adverse effects have been reviewed previously (Etzel, 2003; Hardin *et al*., 2003; Hossain *et al*., 2004; Lai, 2006; Revankar, 2003; Yike and Dearborn, 2004), exciting, new findings have been made concerning *S. chartarum* relative to its environmental dissemination, novel bioactive components, unique cellular and subcellular targets, and molecular mechanisms of action. The goal of this review, rather than reiterate these past reviews or risk assessments, is (1) to highlight effects of both *Stachybotrys* and its bioactive constituents in experimental models within the context of these new advances and (2) to relate these findings to possible adverse effects in humans and future research strategies for resolving this formidable public health enigma.

INFECTIVITY

Stachybotrys chartarum is not recognized to be an infectious respiratory pathogen. Relatively few investigations have focused on *S. chartarum*'s ability to survive or proliferate in the lung (Yike *et al*., 2003) and, indeed, most recent studies on toxic and inflammatory effects of its spores in animals do not describe their viability (Flemming *et al*., 2004; Mason *et al*., 1998, 2001; Nikulin *et al*., 1996, 1997; Rand *et al*., 2003; Rao *et al*., 2000a,b; Rosenblum *et al*., 2006). Ochiai *et al*. (2005) observed that survival of *S. chartarum* spores in alveolar macrophages *in vitro* is greater than that of the prototypical fungal respiratory pathogen *Aspergillus fumigatus* relative to phagocytosis, killing, and germination inhibition. However, the same study found that following acute or short-term repeated intranasal instillation of adult mice, *S. chartarum* spores are unable to germinate and are effectively cleared from the lung after 7 days. The primary histological effect for the acute single exposure was intra-alveolar infiltration by neutrophils, whereas short-term repeated exposure caused eosinophilic infiltration of alveoli and perivascular tissues.

In contrast to adult mice and rats, germination is observed in the lungs of 4-day-old rat pups instilled with viable *S. chartarum* spores along with pulmonary inflammation with hemorrhagic exudates (Yike *et al*., 2003). Viable spores were also more injurious to rat pups than nonviable spores. Histologic evaluation revealed that neutrophilic inflammation and interstitial pneumonia with poorly formed granulomas occurred concomitantly with budding spores and fungal hyphae. Despite germination and outgrowth, *S. chartarum* still did not establish an effective infection in this study. Thus, although *S. chartarum* is largely deemed to be noninfectious, limited opportunistic outgrowth and release of bioactive products in extremely young or immunosuppressed animals might exacerbate the effects of inhaled spores.

ALLERGENICITY AND ASTHMA INDUCTION

Induction of allergenic responses to *S. chartarum* is an important consideration because a strong deleterious response in an individual could be evoked after exposure to even a low concentration of this mold in the indoor environment. Asthma is a complex chronic respiratory disorder characterized by reversible airway constriction and airway hyperresponsiveness. The induction of allergic asthma is a special concern because incidence of this disease has increased dramatically over the past three decades in industrialized nations, particularly among children (Stafford *et al*., 2003). Molds have been associated with the exacerbation of asthma even though their contribution to the induction of allergic asthma is less certain. It has been estimated that there are 21.8 million people with asthma in the United States and that approximately 4.6 (2.7–6.3) million cases can be attributed to dampness and mold exposure in the home (Mudarri and Fisk, 2007). The allergenic capacity of *Stachybotrys* or any mold must be considered in the context of the myriad of immune mediated/related effects associated with fungal exposure, including innate inflammatory/irritant responses, adjuvant effects, and other antigen-specific

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hypersensitivity responses. Of critical importance is whether *Stachybotrys* has the potential to cause Type I hypersensitivity or IgE-mediated responses.

A major challenge in evaluating the contributions of *Stachybotrys* in allergic asthma is that data on mold species-specific associations in epidemiological studies are limited or are complicated by the presence of multiple molds and other agents. An additional complicating factor is the cross-reactivity among some mold antigens, making the identification of the sensitizing mold difficult at best. Other challenges to the identification and characterization of fungal allergens are the inherent complexity of environmental samples and the variety of assessment methods (Burge, 2002; Dillon *et al*., 1999; Levetin and Horner, 2002). Exposure assessments have rested largely on viable fungal spore counts, however, exposure to mycelial fragments in the submicron particle-size range is not only possible (Brasel *et al*., 2005a; Cho *et al*., 2005), but might be sufficient to induce allergic responsiveness in a genetically predisposed population.

Despite a self-reported association of *Stachybotrys* with allergic symptoms in persons exhibiting DBRI (Cooley *et al*., 1998), two investigations of *Stachybotrys*-contaminated buildings failed to find a significant difference between the presence of IgE or IgG antibodies to this fungus in case and control individuals (Johanning *et al*., 1996) or a relationship of these antibodies to the presence of human disease (Hodgson *et al*., 1998). In studies using a histamine release test (HRT), one found functional *S. chartarum*–specific IgE in DBRI patients without Type I allergic disease indicative of sensitization (Larsen *et al*., 1997). The other study found a significant association between a positive HRT (*S. chartarum* specific) and DBRI but not between positive HRT and self-reported hay fever or asthma (Lander *et al*., 2001).

Several laboratories have employed human serum to identify *S. chartarum* proteins that bind IgE. Both 50- and 65-kDa proteins that might act as allergens were detectable in *S. chartarum* culture extracts (Raunio *et al*., 2001). Two other proteins (34 and 52 kDa) were found to be reactive with pooled anti-*S. chartarum* human IgE sera (Barnes *et al*., 2002). Recently, Xu *et al*. (2007) have also isolated a 34-kDa protein that is antigenic in humans. Karkkainen *et al*. (2004) identified eight potential allergenic proteins with human IgE anti-*S. chartarum* sera. Greater than 80% of the human sera (< 50% of control sera) reacted with 33-, 48-, and 50-kDa proteins. Subsequent analyses of the 48-kDa proteins demonstrated homology with fungal cellulases. Additionally, these sera recognized 48- and 50-kDa proteins from seven other molds suggesting shared antigens. Taken together these studies indicate that some humans have developed antibodies that are reactive with *S. chartarum* proteins. Whether these antibodies are a result of direct exposure to *S. chartarum* or are the result of cross-reactivity among mold antigens is not always clear. Although, the presence of *S. chartarum* reactive IgE does not always indicate the presence of allergic disease, it does indicate the possibility that exposure could trigger an allergic event without prior exposure (to S. Chartarum due to cross reactivity with another allergon).

Animal models of allergic airway disease provide further insight into the allergic potential of molds or their previously identified IgE reactive proteins. Several animal studies have assessed the allergic potential of *S. chartarum*. Korpi *et al*. (2002) immunized two mouse strains (BALB/c, National Institutes of Health) with a nontoxic *S. chartarum* mycelium extract in alum resulting in significantly elevated antigen-specific IgG_1, IgG_{2a}, and IgA, as well as, total IgE compared with nonimmunized controls. Subsequent aerosol exposure to *S. chartarum* extract resulted in mild inflammatory sensory irritation with changes in pulmonary function as measured by plethysmography in mold-immunized and naïve mice. This effect was seen neither in ovalbumin-challenged nor in phosphate-buffered saline aerosol-challenged mice even when mice were previously immunized with ovalbumin.

Stachybotrys chartarum extract (from spores and mycelium) can induce antigen nonspecific lung injury and inflammation as demonstrated by increased lung permeability, bronchoalveolar lavage fluid (BALF) total protein, cell damage (BALF lactate dehydrogenase activity), and BALF total cell influx, including neutrophil influx in BALB/c mice (Viana *et al*., 2002). This experimental design included multiple intratracheal aspiration extract exposures (Fig. 1A) and evoked responses indicative of allergen-specific immune responses including elevated BALF interleukin (IL)-5 and total and antigen-specific serum IgE. Furthermore, this study evaluated immediate airway reactivity following fungal extract challenge and hyperresponsiveness to nonspecific methacholine challenge using whole-body plethysmography. The results demonstrated the development of a significantly increased airway response following multiple extract exposures (Fig. 1B) as well as a significant increase in the response to nonspecific stimuli (methacholine) in sensitized mice (Fig. 1C). The conclusion from this work is that *S. chartarum* has the capacity to induce responses in mice that are characteristic of human allergic asthma. Subsequently, these investigators have compared the responses of BALB/c mice exposed to *S. chartarum*, *Metarhizium anisopliae* (a biopesticide), or house dust mite. These data suggest that *S. chartarum* extract induced dose-dependent increases in BALF neutrophil and eosinophil influx and serum total IgE three days after the final respiratory exposure (Figs. 1D and 1E). However, the *S. chartarum* extract was less potent than the two other extracts at inducing human allergic asthma-like responses (unpublished data).

In contrast to the extract studies, anti-*S. chartarum* antibodies were not detectable in sera from mice exposed repeatedly to the intact spores via the nose (Leino *et al*., 2003; Nikulin *et al*., 1996, 1997). Besides the form of antigen, differences in results across allergenicity studies might be a consequence of animal susceptibility, route of exposure, and/or dose. Nikulin *et al*. (1996, 1997) used outbred NMRI strain

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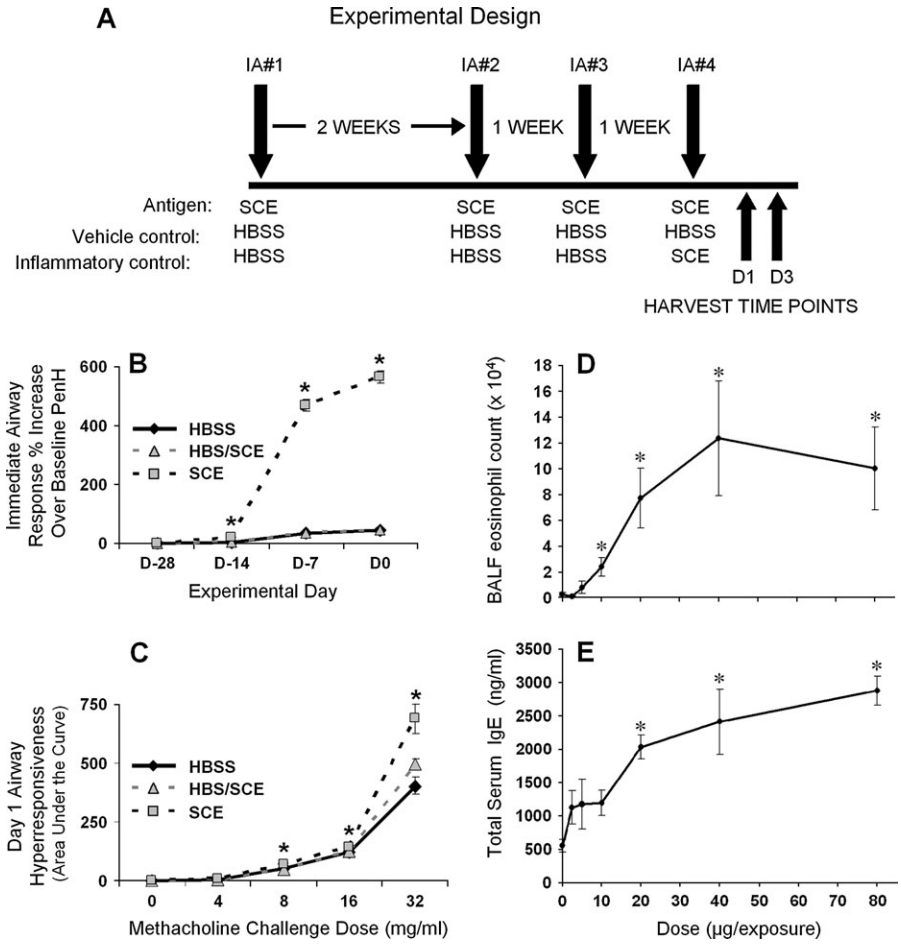

FIG. 1. *Stachybotrys chartarum* extract induces airway allergic responses in the mouse. Top panel (A) a summarieos experimental design for BALB/c mice exposure to *S. chartarum* extract (SCE), Hank's Balanced Salt Solution (HBSS, vehicle control) or HBS/SCE (inflammatory control). Left panels show (B) percent increase over baseline enhanced pause (PenH) immediately following exposure and (C) airway response to the nonspecific stimulus methacholine 1 day after the last SCE exposure. Asterisk indicates significant difference compared with HBSS control at $p < 0.05$ ($n = 6$). Right panels show dose-dependent (D) BALF eosinophil and (E) total serum IgE increases 3 ds after the fourth *S. chartarum* aspiration exposure. Asterisk indicates significant difference compared with 0 dose control at $p < 0.05$ ($n = 6$). Panels B and C were modified with permission from *Toxicological Sciences* (Viana *et al.*, 2002).

mice in their studies. In an outbred population it is more difficult to assess allergic potential compared with a population that is genetically predisposed to allergy-like responses analogous to the human atopic population such as inbred BALB/c mice. Although Leino *et al.* (2003) used the BALB/c mouse model, neither anti-*S. chartarum* IgG (biomarker of exposure) nor IgE was detected. However, there are several differences between this study and the ones using *S. chartarum* extract (Viana *et al.*, 2002). In the Leino *et al.* study, spores a from single strain of *S. chartarum* were instilled by the intranasal route. In the Viana *et al.* study, a pooled extract of both spores and mycelium from five isolates was instilled by intratracheal aspiration. Furthermore, culture conditions/media used in the two studies were different. The difference in culture conditions could enhance any intrinsic differences in the ability of various *S. chartarum* strains/isolates to induce allergic responses. Additionally, the antigen/allergen content of spores and mycelium may be very different. Therefore, the differences in the two studies could lead to differing antigenic profiles. Another factor is route of exposure. The intranasal exposure used by Leino *et al.* (2003), a natural route for mold exposure, might have facilitated rapid spore clearance leaving insufficient time for immunologically inductive amounts of water-soluble proteins (allergens) to be leached from the spores. The more respirable fragments of spores and mycelium may be the source of allergens.

Rosenblum *et al.* (2002) tested the hypothesis that underlying allergic inflammation in BALB/c mice contributes to increased susceptibility to induction of pulmonary inflammation and injury by *Stachybotrys* spores. Surprisingly, ovalbumin-induced airway inflammation produced a protective effect against specific *S. chartarum*–induced pulmonary responses. The authors suggested that allergen-induced mucous hypersecretion increased tolerance to *S. chartarum* in the sensitized

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mice by creating a more effective barrier to spores or its toxins. Furthermore, ovalbumin induction of T helper 2 (Th2)–mediated responses in airways might have counterbalanced the robust T helper 1 (Th1)–mediated inflammatory responses. These workers have further determined that Th2-biased BALB/c mice were much more responsive to *S. chartarum* spores than Th1-biased C57BL/6J mice (Rosenblum *et al.*, 2006). They speculated that differences in Th1- and Th2-biased immune responses among persons resulting from genetic predisposition or previous environmental exposures could contribute to the varied responses observed among individuals exposed to identical moldy environments. It might also be predicted that young infants, who have a Th2-biased immune response, would be less efficient at clearing *S. chartarum* spores and therefore generate an exaggerated inflammatory response before the immune host defense system can kill or clear the spores. Therefore, young infants might be more susceptible to subsequent allergic airway disease.

Taken together, although epidemiological investigations have yet to demonstrate a clear association between *S. chartarum* exposure and allergy/asthma, human serum studies have identified IgE specific for *S. chartarum* antigens, which suggest that fungus can induce sensitization. Animal models confirm the potential for *S. chartarum* exposure to induce proinflammatory responses and also to evoke responses characteristic of human allergic asthma. Although these studies suggest the capacity of *S. chartarum* to induce sensitization, numerous questions remain to be answered before the allergy risks associated with human exposure to *S. chartarum* are fully understood.

TRICHOTHECENE TOXICITY

Approximately one-third of the *S. chartarum* isolates from the United States and Europe represent a chemotype that produces macrocyclic trichothecene mycotoxins (Andersen, 2002). The capacity of *S. chartarum* to produce these biologically potent trichothecenes has been of great public health concern and has led to this fungus being colloquially termed "toxic black mold."

Stachybotrys and Animal Illness

Animal illnesses have been described following ingestion or inhalation of feed contaminated with *Stachybotrys* (Aizenman, 1993; Aizenman and Kudlai, 1978; Akkmeteli, 1977; Bojan *et al.*, 1976; Harrach *et al.*, 1983; Schneider *et al.*, 1979; Sudakin, 2000; Tantaoui-Elaraki *et al.*, 1994), with adverse effects occurring in two phases. Elevated body temperature, listlessness, epistaxis, and intermittent hemorrhagic diarrhea occur during the first phase. The second phase consists of a progressively worsening anemia, leukocytopenia, hemorrhage, and pulmonary congestion. These adverse effects are highly consistent with the known toxic effects of trichothecene mycotoxins (Ueno, 1984b).

Chemistry

The trichothecene family contains over 200 structurally related sesquiterpenoid metabolites produced by *Stachybotrys*, *Myrothecium*, *Fusarium*, and other fungi (Grove, 1988, 1993, 2000, 2007). All trichothecenes have a common 9, 10 double bond and a 12, 13 epoxide group, but extensive variation exists relative to ring oxygenation patterns. From a public health perspective, trichothecenes belonging to three structural groups appear to be most important (Fig. 2). Common to contaminated foods are the Type A trichothecenes, which have isovaleryl, hydrogen, or hydroxyl moieties at the C-8 position (e.g., T-2 toxin), and the Type B trichothecenes, which have a carbonyl group at the C-8 position (e.g., deoxynivalenol or "vomitoxin"). Macrocyclic or Type D trichothecenes have a cyclic diester or triester ring linking C-4 to C-15 (e.g., satratoxins or roridins) and are the type primarily produced by *Stachybotrys*.

Trichothecene Dissemination and Delivery

Macrocyclic trichothecenes occur in the outer plasmalemma surface and the inner wall layers of *Stachybotrys* conidiospores (Gregory *et al.*, 2004). Dry spores are readily aerosolized and have a respirable mean aerodynamic diameter of approximately 5 μm (Sorenson *et al.*, 1996; Yike and Dearborn, 2004). When suspended in aqueous buffer, *S. chartarum* spores release trichothecenes within minutes (Yike, personal communication). Similar rapid release appears to occur in rat lungs (Yike *et al.*, 2005). In addition, nonviable, fine ($\leq$ 1 μm in diameter) airborne particulates containing satratoxins can be released from cultures (Brasel *et al.*, 2005a). Thus, it is possible that spores, fragmented mycelia and, perhaps, decomposed substrate could facilitate trichothecene dissemination, delivery, and release to the respiratory tracts of individuals in *Stachybotrys*-contaminated buildings. Trichothecenes have been detected in air samples taken in indoor air quality investigations suggesting that these compounds can be aerosolized on spores or small particles (Brasel *et al.*, 2005a; Charpin-Kadouch *et al.*, 2006; Vesper *et al.*, 2000a).

Limited information exists on the kinetics of trichothecene uptake and disposition *in vivo*. Immediately following intratracheal instillation of rat pups with *S. chartarum* spores (equivalent to 100 μg of satratoxin G [SG]/kg body weight [bw]), approximately 5% of SG was recoverable in BALF (Yike and Dearborn, 2004). Toxin recovery in BALF decreases to 0.34% after 30 min and declines rapidly thereafter. Although 0.2% of SG was recoverable in ethanol extracts of whole blood of these pups immediately after exposure, the toxin was undetectable after 15 min.

In lung tissues of mice instilled with toxic *S. chartarum* spores, satratoxin was immunochemically detectable in macrophage lysosomes as well as along the inside of the nuclear

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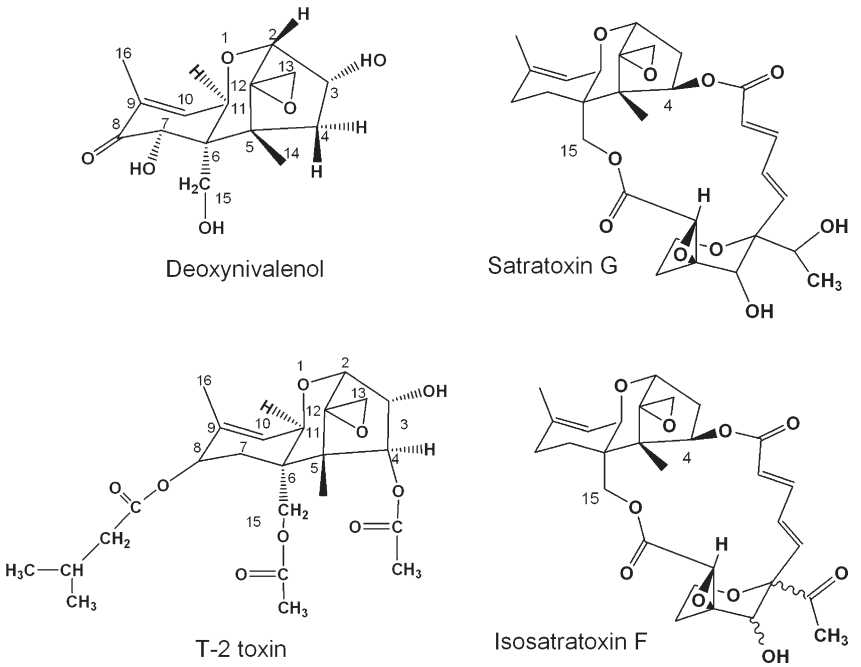

FIG. 2. Trichothecene mycotoxins. Chemical structures of representative Type D macrocyclic (SG and isosatratoxin F) and Type A (T-2 toxin), Type B (deoxynivalenol) trichothecenes.

membrane in nuclear heterochromatin and the rough endoplasmic reticulum (Gregory *et al*., 2004). Modest labeling was also found in the nuclear heterochromatin and rough endoplasmic reticulum of alveolar type II cells, whereas there was little evidence of toxin accumulation in neutrophils, fibroblasts, or other cells associated with the granuloma tissues surrounding spores or mycelial fragments.

Overall, these limited studies suggest that delivery and release of trichothecenes from *S. chartarum* spores is indeed possible and that subsequent absorption and metabolism is extremely rapid. Furthermore, alveolar macrophages are likely to play an important role in sequestration of the *S. chartarum* spores and their trichothecene mycotoxins.

Molecular Effects of Trichothecenes

Trichothecenes can mediate toxicity by interfering with ribosome function, activating stress responses, and forming covalent adducts. Trichothecenes bind to the eukaryotic ribosomes and prevent polypeptide chain initiation or elongation in cell-free models (Bamburg, 1983; Carter and Cannon, 1977; Ueno, 1984a). Partial translational inhibition by trichothecenes is demonstrable both in cell culture and experimental animals (Azcona-Olivera *et al*., 1995; Thompson and Wannemacher, 1986). In addition to inhibiting translation, trichothecenes can simultaneously activate p38, Jun N-terminal Kinase (JNK) and extracellular signal-regulated kinase (ERK) mitogen-activated protein kinases (MAPKs) *in vitro* and *in vivo* (Chung *et al*., 2003d; Moon and Pestka, 2003; Moon *et al*., 2003; Yang *et al*., 2000; Zhou *et al*., 2003a,b, 2005a,b) via a process referred to as "ribotoxic stress" (Iordanov *et al*., 1997).

Trichothecene-induced MAPK activation mediates both proinflammatory cytokine upregulation as well as apoptosis (Pestka *et al*., 2004; Zhou *et al*., 2003b, 2005b). In cell cultures, macrocyclic trichothecenes are 10–100 times more potent than Type A or Type B trichothecenes at activating MAPKs, impairing leukocyte proliferation, or inducing apoptosis (Chung *et al*., 2003b,c; Hughes *et al*., 1989, 1990; Pestka and Forsell, 1988; Shifrin and Anderson, 1999; Yang *et al*., 2000). In macrophages, at least two kinase types, double-stranded RNA-activated protein kinase (PKR) and the Src family kinases, have upstream roles in MAPK activation by the Type B trichothecene, deoxynivalenol (Pestka *et al*., 2004). The potential for macrocyclic trichothecenes to similarly mediate via these intracellular signaling pathways merits further investigation.

Macrocyclic trichothecenes or other factors from *S. chartarum* might also have the capacity to damage DNA. McCrae *et al*. (2007) determined that aqueous extracts of a trichothecene-producing *S. chartarum* spores for at least 15 min to 14 h, induced DNA fragmentation in developing rat lung fibroblasts. Microarrays were used to assess effects of a methanol extract of a trichothecene-producing strain of *S. chartarum* prior to and after the onset of apoptosis in the murine alveolar macrophage cell line MH-S (Wang and Yadav, 2007). The extract modulated expression of genes related to cell growth, proliferation and death, inflammatory/immune response, genotoxic stress, and oxidative stress, as well as

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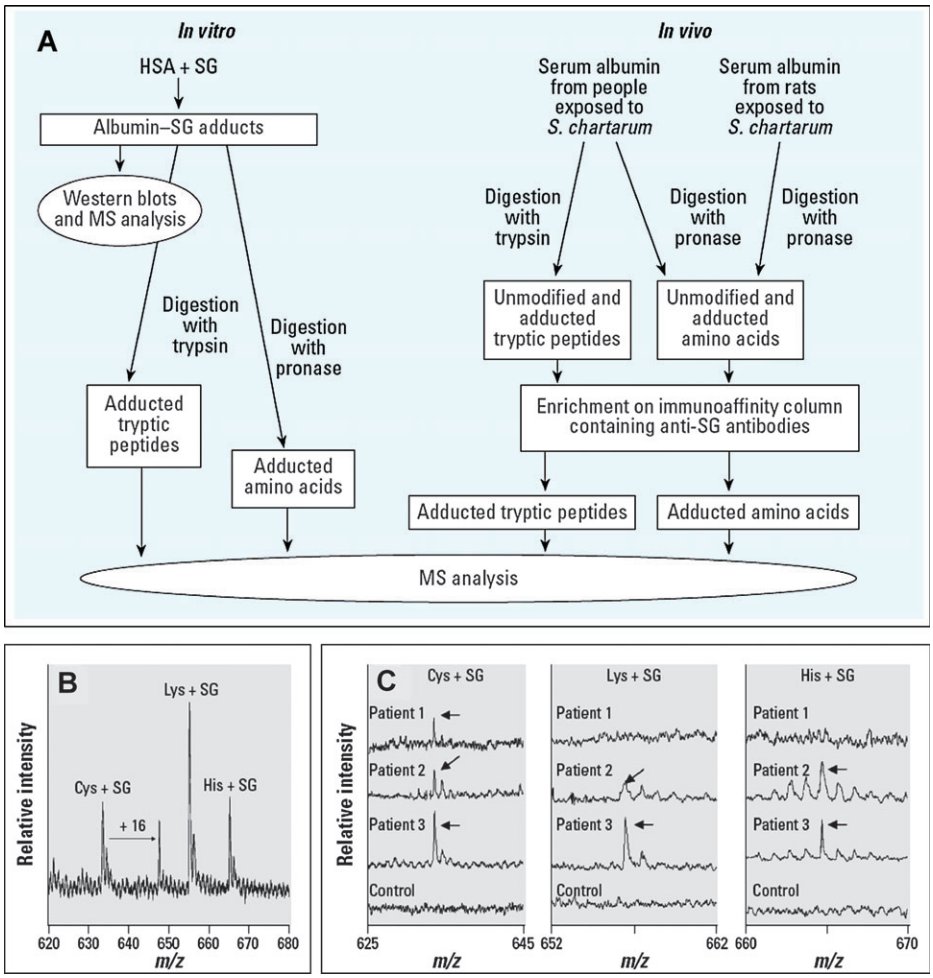

FIG. 3. SG forms adducts with human serum albumin (HSA) *in vitro* and *in vivo*. Top panel (A) indicates experimental design for generation and detection of SG protein adducts. Lower panels show representative matrix-assisted laser desorption-time of flight (MALDI–TOF) spectra of amino acid–SG adducts in pronase digests of (B) recombinant HSA *in vitro* incubation mixtures and of (C) HSA from *S. chartarum*–exposed and control patients. Reproduced with permission from *Environmental Health Perspectives* (Yike *et al*., 2006).

signal transduction pathways involving MAPK, nuclear factor-kappaB, tumor necrosis factor (TNF)-α, and p53. Particularly notable was upregulation of DNA damage-responsive and DNA repair genes early in the treatment, which are suggestive of genotoxic stress. In a follow-up study, extract-induced apoptosis in MH-5 cells was observed to precede DNA damage (Wang and Yadav, 2006). Methanol extract–treated macrophages accumulated p53, as well as having time-dependent activation of JNK and p38. Pharmacologic inhibition of either p38 or p53 partially inhibited extract-induced apoptosis, whereas blocking of JNK with a chemical inhibitor had no effect. These authors proposed that both p38- and p53-mediated signaling events occurred in *S. chartarum*–induced apoptosis of alveolar macrophages. Overall, because extracts were used in these studies and toxin content was not measured, it is not clear what the relative contributions of trichothecenes or other extracted compounds were to the effects observed in these studies.

In addition to evoking stress responses, SG, a macrocyclic trichothecene isolated from *S. chartarum* cultures, has the potential to form adducts with cellular macromolecules. Early studies revealed that immunoreactive SG is detectable in murine tissue sections and in BALF cells obtained from mice exposed to the toxin (Gregory *et al*., 2004). Satratoxin epitopes were still detectable in lung sections after extensive washing in organic solvents thus suggesting that the toxin bound tightly to cell components. When human serum albumin and SG were coincubated and immunoblotted with anti-satratoxin antibodies, adducts were produced in a concentration-dependent fashion that was strongly indicative of a covalent linkage between the toxin and protein (Yike *et al*., 2006) (Fig. 3A). Mass spectrometry revealed that up to 10 toxin molecules were bound per albumin molecule in this model. Sequence analysis indicated that lysyl, cysteinyl, and histidyl residues were involved in the formation of adducts (Fig. 3B). The potential of satratoxin and other macrocyclic trichothecenes to covalently

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bind proteins and possibly other macromolecules has major implications relative to their absorption, metabolism, distribution, toxicity, and potential allergenicity. Furthermore, these data suggest that such covalent adducts can be used as biomarkers of human exposure (Fig. 3C).

Pulmonary Airway Toxicity

Early investigations in mice provided qualitative evidence that soluble toxins were critical for adverse pulmonary effects of *S. chartarum* spores. Nikulin *et al.* (1996), using 5-week-old mice, compared the pulmonary effects of intranasal exposure to "toxic" and "nontoxic" *S. chartarum* isolates which differed by 13,000-fold in spore extract cytotoxicity. Only half of the animals treated with toxic spores survived with the survivors showing a dramatic loss in body weight. Mice receiving toxic *S. chartarum* spores exhibited more extensive lung inflammation, with hemorrhagic exudates than those receiving nontoxic spores. Analogous lung inflammation and histopathology were observed in mice subjected to six repeated exposures of 10^3 and 10^5 toxic *S. chartarum* spores but not in mice receiving comparable doses of nontoxic spores at these lower doses (Nikulin *et al.*, 1997). It should be noted that these studies were conducted prior to recognition of macrocyclic trichothecene- and atranone-producing chemotypes, therefore, it is possible that these toxic and nontoxic isolates corresponded to these two groups, respectively.

When the pulmonary effects of intratracheal exposure to toxic *S. chartarum* spores (3×10^5–3×10^7/kg bw) were assessed in 10-week-old rats, dose-dependent increases in lactate dehydrogenase, albumin, hemoglobin, myeloperoxidase, and leukocyte differential counts were observed in BALF after 24 h (Rao *et al.*, 2000a,b). These effects were undetectable in rats treated similarly with methanol-extracted spores, suggesting that pulmonary inflammation and injury corresponded to methanol-soluble toxins within the spores. Because toxicity of the spores used in these studies was based only on the shrimp bioassay, the toxin identity, and quantity are not known.

Murine alveolar type II cells and alveolar macrophages are extremely sensitive to intratracheally instilled *Stachybotrys* spores (Rand *et al.*, 2002) and these effects could be mimicked using pure isosatratoxin F. Both spores and isosatratoxin F exposure evoked marked changes in pulmonary surfactant synthesis and secretion as well as phospholipid targeting to pulmonary surfactant pools in mice. Both preparations also altered the activity of convertase, an enzyme capable of converting surfactant fractions from surface active to those metabolically used (Mason *et al.*, 2001). This activity might have been associated with alveolar type II cell damage (McCrae *et al.*, 2001). *Stachybotrys chartarum* spores and toxin have been shown to depress disaturated phosphotidylcholine, which is the major phospholipid responsible for maintaining surface-tension properties of lung surfactant (Sumarah *et al.*, 1999). Modulation of another key enzyme, CTP:cholinephosphate cytidylyltransferase, in the phosphotidylcholine synthesis pathway, might also contribute to that depression (Hastings *et al.*, 2005).

The dose-dependent toxicity of a single tracheal instillation of up to 8×10^8 nonviable, highly toxic *S. chartarum* spores/kg bw was assessed in 4-day-old rat pups by measuring survival, growth, lung histopathology, and respiration (Yike *et al.*, 2002). Because there is approximately 1 pg of SG equivalent per spore, the concentrations of macrocyclic trichothecenes are ~1mM within the spore. At 72-h postinstillation (PI), the LD_{50} dose was 2.7×10^8 spores/kg bw, with dead pups exhibiting extensive lung hemorrhage. Altered pulmonary function (decreased respiratory rate and higher tidal volume) correlated with increased pulmonary resistance seen in rats exposed to 1.1×10^8 spores/kg bw but having a low mortality rate (2%). Lungs of these rats exhibited hemorrhage, hemosiderin-laden macrophages, and inflammation as evidenced by thickened alveolar septa that were infiltrated with lymphocytes and other mononuclear cells. After 3 days, BALF contained elevated numbers of macrophages, lymphocytes, and neutrophils as well as increased IL-1β and TNF-α. At 8 days PI, inflammatory cells number declined significantly. Because ethanol-extracted (i.e., trichothecene-free) spores had negligible effects on these aforementioned endpoints, it was concluded that these mycotoxins were required for the hemorrhagic and inflammatory responses in the rat pup.

Because protein factors in *Stachybotrys* might act in addition to trichothecenes, the effects of intact, autoclaved, and ethanol-extracted spores on the lungs of infant rats were compared (Yike *et al.*, 2005). Alveolar space, defined as percentage of air space in the lung parenchyma, after 24 h were 42% for intact spores, 51% for ethanol-extracted spores, 57% for autoclaved spores, and 61% for PBS-treated controls. Effects of the different spore preparations on inflammatory cells, cytokine, and protein concentrations in the BALF followed the rank order: intact > autoclaved > extracted. The difference observed between autoclaved (100% trichothecene activity, denatured/enzymatically inactive proteins) and intact (100% trichothecene activity, unaltered/released proteins) spores suggest that in addition to trichothecenes, *S. chartarum* proteins contributed to the inflammatory response.

In contrast to the aforementioned studies, no significant differences were observed in the induction of the proinflammatory cytokines IL-1β, IL-6, and TNF-α in the BALF of mice exposed repeatedly to the satratoxin-producing and nonproducing isolates (Leino *et al.*, 2003). The effects of *S. chartarum* spores were similar for both types of isolates, although one chemokine, CXCL5/LIX, showed significantly higher messenger RNA (mRNA) levels after exposure to the satratoxin-producing isolate. BALF compositions were also similar from mice exposed to low doses of high trichothecene-producing (JS58-17) and nontoxic (JS58-06) isolates of *S. chartarum* spores (Flemming *et al.*, 2004). Thus, fungal components other than trichothecenes might have the capacity to evoke *Stachybotrys*-induced pulmonary inflammation. These are discussed later in this review.

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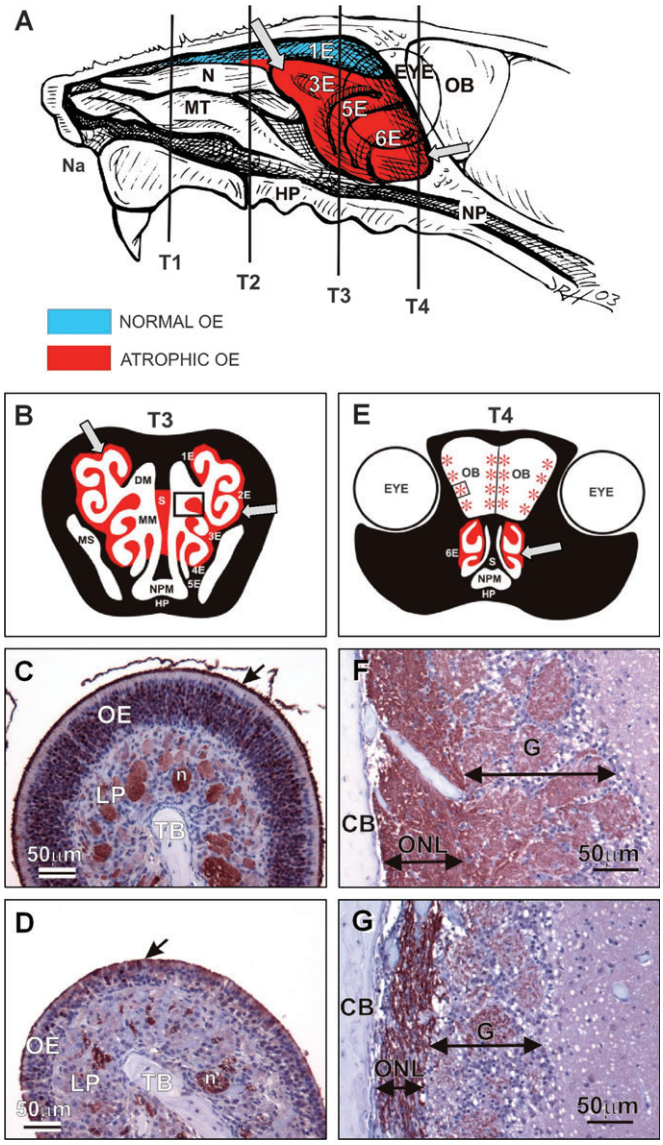

FIG. 4. SG exposure depletes olfactory sensory neurons (OSNs) in olfactory epithelium (OE) and olfactory bulb (OB). Abbreviations: CB, cranial bone; DM, dorsal medial meatus (airway); E, ethmoid turbinate; G, glomerular layer; HP, hard palate; L, lateral meatus; MM, middle meatus; MS, maxillary sinus; MT, maxilloturbinate; N, nasoturbinate; Na, naris; NP, nasopharynx; NPM, nasopharyngeal meatus; ONL, olfactory nerve layer; S, nasal septum; and TB, turbinate bone. Top panel (A) is diagrammatic representation of the right nasal passage with the nasal septum removed, exposing the luminal surfaces of nasal turbinates (N, MT, 1E–6E) projecting from the lateral wall; vertical lines indicate the anterior surfaces of transverse tissue blocks (T1–T4) that were selected for microscopic examination. Panels immediately below are cross-sectional views of T3 (B) and T4 (E). Red areas indicated by open arrows in (A), (B), and (E) represent OE that exhibited SG-induced apoptosis and atrophy 1–7 days after single acute SG nasal instillation. (E) T4 nasal section containing OB and sites of ONL and G layer atrophy induced by SG exposure (asterisks). (C, D) Light photomicrographs of OE lining 3E in T3 nasal section (square in B) from mice 1 day PI with (C) saline vehicle alone or (D) SG (500 µg/kg bw). Sections were immunohistochemically stained for OMP, a marker for mature OSNs, as indicated by dark brown stained areas. Arrows in (C) and (D) indicate OMP-stained cilia along the airway surface. There is marked loss of OMP-stained OSNs and cilia in (D) compared with (C). (F, G) Light photomicrographs of olfactory nerve layer (ONL) in OB located in T4 nasal section (square in E) of mice 7 days PI with (F) saline vehicle alone or (G) SG (500 µg/kg bw). OMP detection (dark brown stain) reveals depletion of OSNs in ONL and G layer of the OB in SG-treated mice. Reproduced with permission from *Environmental Health Perspectives* (Islam *et al.*, 2006).

Nasal Airway Toxicity

In addition to targeting the lung, macrocyclic trichothecenes might affect the upper respiratory tract (e.g., nasal airways). The nose is complex with multiple functions that include conditioning of inhaled air and olfaction (Cole, 1993). Nasal passages function as respiratory tract scrubbers by (1) absorbing water-soluble and reactive gases and vapors, (2) trapping inhaled particles, and (3) metabolizing airborne xenobiotics (Harkema *et al.*, 2006). Because the nose is a portal of entry for inhaled air, it is a key target for toxicity by airborne contaminants with the surface epithelial lining being the principal site of toxic injury. The four distinct surface epithelia lining mammalian nasal passages include: (1) squamous epithelium, (2) ciliated, pseudostratified, respiratory epithelium, (3) nonciliated transitional epithelium, and (4) olfactory epithelium (OE) (Harkema *et al.*, 2006). Substantial deposition (> 20–50%) of either very large (> 5 µm mean aerodynamic particle diameter, such as a fungal spore) or very small particles (< 10 nm in diameter; nanoparticles) occurs in the nasal airways of humans and laboratory animals following nasal inhalation (Cheng *et al.*, 1990, 1997a,b; Yeh and Schum, 1980). Modeling studies confirm that spores and other large *S. chartarum* fragments (≥ 5 µm in diameter) would deposit in the human nose (Cho *et al.*, 2005). Thus, the mucosal tissue lining the nasal airway could be an important target for *Stachybotrys*.

Acute intranasal exposure to SG specifically induced apoptosis of olfactory sensory neurons (OSNs) with atrophy of the OE in adult C57BL/6J mice (Islam *et al.*, 2006) (Figs. 4A–D). These effects were dose-dependent with the no-observed adverse effect level (NOAEL) and the lowest-observed adverse effect level (LOAEL) at 24-h PI being 5 and 25 µg/kg bw SG (equivalent to 5 and 25 $\times$ 10^6 spores/kg bw), respectively. Elevated expressions of the proapoptotic genes Fas, FasL, p75NGFR, p53, Bax, caspase-3, and CAD in the OE-lined ethmoid turbinate were found to correspond to OSN apoptosis. Time-course studies with a single instillation of SG (500 µg/kg bw) indicated that maximum atrophy of the OE occurred at 3 days PI. Exposure to lower doses (100 µg/kg bw) for 5 consecutive days resulted in similar OE atrophy and OSN apoptosis, suggesting that in the short term, these effects were cumulative. SG also induced an acute, neutrophilic rhinitis as early as 24-h PI. Elevated mRNA expression for TNF-α, IL-6, IL-1β, and the chemokine macrophage-inflammatory protein-2 (MIP-2) was detected at 24-h PI in both the ethmoid turbinates of the nasal airways and the adjacent

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olfactory bulb of the brain. Marked atrophy of the olfactory nerve and glomerular layers of the olfactory bulb was also detectable by 7 days PI along with mild neutrophilic encephalitis (Figs. 4E–G).

In a follow-up study, the kinetics of nasal neurotoxicity and inflammation in adult C57BL/6J mice (Islam *et al*., 2007) was assessed after a single intranasal instillation of roridin A (250 and 500 μg/kg bw), a commercially available macrocyclic trichothecene used as a SG surrogate. As observed for SG, roridin A induced apoptosis OSNs at 1 day PI causing marked atrophy of OE and the olfactory nerve layer (composed of OSN neurons) of the olfactory bulbs in the frontal brain that was maximal at 3 days PI. In the ethmoid turbinates, upregulated mRNA expression of the proapoptotic gene FAS, the proinflammatory cytokines TNF-α, IL-6, IL-1, and the chemokine MIP-2 were observed from 6- to 24-h PI, whereas expression of several other proapoptotic genes (PKR, p53, Bax, and CAD) were detectable only at 24-h PI. Thus, macrocyclic trichothecene-induced FAS and proinflammatory cytokine expression were evoked prior to OSN apoptosis and OE atrophy. Overall, these findings suggest that neurotoxicity and inflammation within the nose and brain are potential adverse human health effects of exposure to SG and roridin A (Fig. 5). It will be critical to ascertain whether chronic low dose exposure to macrocyclic trichothecenes or spores from the trichothecene-producing *Stachybotrys* chemotype will evoke similar toxicity.

Macrocyclic trichothecenes might drive both extrinsic (death receptor-mediated) and intrinsic (mitochondrial-mediated) apoptotic pathways in OSNs via the ribotoxic stress response (Yang *et al*., 2000). Notably, these toxins induced genes in nasal turbinates that have previously been associated with death receptor-mediated OSN apoptosis including TNF-α, Fas, FasL, and p75NGFR (Cowan and Roskams, 2002) as well as resultant downstream apoptotic genes p53 (Huang *et al*., 2000), Bax (Ge *et al*., 2002), and caspase-3 (Cowan and Roskams, 2004). Relative to the intrinsic pathway, the trichothecene deoxynivalenol has been previously shown to directly induce p38-mediated mitochondrial-dependent caspase-3 activation and apoptosis in cloned macrophages (Zhou *et al*., 2005a). Furthermore, satratoxin H–induced caspase-3 activation and apoptosis in the PC12 neural cell model have recently been reported to be both p38 and JNK dependent (Nusuetrong *et al*., 2005).

Role of Trichothecenes in Stachybotrys Pathophysiology

Taken together, macrocyclic trichothecenes are extremely potent biological modulators that cause numerous pathophysiologic effects in experimental animals and that could conceivably contribute to adverse human health effects.

ATRANONE TOXICITY

In addition to trichothecenes, a second mycotoxin family might contribute to adverse health effects associated with

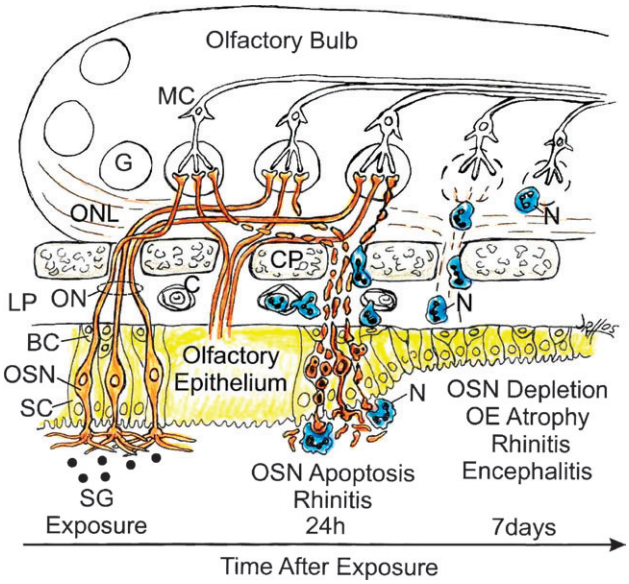

FIG. 5. Diagrammatic representation of sequential SG-induced pathology in olfactory epithelium (OE) and olfactory bulb (OB) after single acute exposure. Abbreviations: BC, basal cells; C, capillary in LP; CP, cribiform plate; G, glomerular; LP, lamina propria; MC, mitral cell; N, neutrophil; ON, olfactory nerve; ONL, olfactory nerve layer; OSN; olfactory sensory neuron and SC, sustentacular cells. OE is shown in yellow, normal OSN in orange, and neutrophils phagocytosing apoptotic OSNs in blue. Reproduced with permission from *Environmental Health Perspectives* (Islam *et al*., 2006).

S. chartarum isolates that do not produce macrocyclic trichothecenes typically belong to a second, genetically distinct chemotype that produces a unique class of metabolites related to the dollabellane diterpenes known as atranones (Hinkley *et al*., 1999, 2000; Jarvis, 2002; Peltola *et al*., 2002) (Fig. 1). Seven atranones, designated A through G, have been isolated from *Stachybotrys* cultures (Hinkley *et al*., 2000). It has been speculated that cytotoxicity of *S. chartarum* isolates is related to satratoxin production, whereas induction of inflammation is due to atranone production (Nielsen *et al*., 2002).

Extracts of atranone-producing *S. chartarum* induce TNF-α and IL-6 production in the RAW 264.7 murine macrophages (Nielsen *et al*., 2002; Peltola *et al*., 2002). The murine lung responses to spores of the atranone-producing *S. chartarum* strain are very different from the lung responses generated by spores from the macrocyclic trichothecene-producing strain (Flemming *et al*., 2004; Hudson *et al*., 2005). The latter evoke a rapid inflammatory response that is sustained for 24-h PI then declines to control values, whereas the atranone-producing chemotype stimulates inflammatory responses lasting up to 96-h PI. It has been postulated that these temporal response differences are attributable to pharmacokinetic properties of the spore-sequestered toxins of these two chemotypes (Flemming *et al*., 2004). However, it was not clear from these studies whether the differences resulted from spore-sequestered atranones alone, or to some other bioactive spore properties such as proteinases or glucans (see below).

Dose–response (0.2, 1.0, 2.0, 5.0, or 20 μg/animal) and time-course (3, 6, 24, and 48 h) effects of intratracheal instillation of

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mice with purified atranones (either A or C) on inflammatory cell and proinflammatory chemokine/cytokine responses were recently examined in mice (Rand *et al.*, 2006). The highest atranone doses (2.0–20 μg toxin/animal, equivalent to 87 and 870 μg/kg bw, respectively), induced significant inflammatory responses manifested by elevated numbers of macrophages and neutrophils as well as increased MIP-2, TNF-α, and IL-6 concentrations in BALF. These results suggest not only that atranones are inflammatory but that various atranones can differ in inflammatory potency as well as toxicokinetics. Atranones might therefore be another contributing inflammatory factor in spores and mycelial fragments of *S. chartarum*.

EFFECTS OF OTHER BIOLOGICALLY ACTIVE PRODUCTS OF *STACHYBOTRYS*

Stachybotrys produces other biologically active products that might, collectively with trichothecenes and atranones, contribute to pathologic effects of this fungus. These include hemolysins, proteinases, glucans, spirocyclic drimanes, and volatile organic compounds.

Hemolysins

Based on the observation that *Stachybotrys* isolates from homes of Cleveland infants suffering from pulmonary hemorrhage exhibited hemolytic activity (Vesper *et al.*, 1999, 2000b), stachylysin, a novel protein and hemolysin, was isolated from the fungus. Stachylysin causes hemorrhage in annelids (Vesper and Vesper, 2002). Immunohistochemical studies have revealed that stachylysin localizes in the inner cell wall of the *S. chartarum* spore (Gregory *et al.*, 2003). Following intratracheal instillation with *S. chartarum* spores, more stachylysin is observed in mouse lung at 72 h than at 24 h suggesting that production/release is a relatively slow process. The authors further observed that stachylysin localizes in phagolysosomes. These data suggest that macrophages might facilitate inactivation of this fungal enzyme as has been previously observed for the hemolysin produced by the pathogen *Aspergillus fumigatus*.

Proteinases

Other proteins could contribute to spore pathotoxicity, most notably, the fungal proteases which can induce inflammation via protease-activated receptors (Kauffman *et al.*, 2000). Proteolytic assays of spore extracts in conjunction with inhibitor studies suggest the presence of serine proteinases in *S. chartarum* spores. The types and quantities of proteases vary with *S. chartarum* isolates and growth conditions (Yike *et al.*, 2007). The best characterized is stachyrase A, a broadly specific serine protease which can hydrolyze several collagens (types I, VI, and X), pulmonary proteinase inhibitors, and several neuropeptides (Kordula *et al.*, 2002). Yike *et al.* (2007) conducted studies in both human tracheal epithelial cells, as well as lungs of rat pups that led to the proposal that *S. chartarum* proteinases contribute to degradation of extracellular matrix proteins either directly via collagenolytic activity or indirectly via changes in the proteinase–antiproteinase balance. These mechanisms might be particularly important in inducing pulmonary hemorrhage in young infants. Figure 6 illustrates some of the pathways that might be involved in lung injury in infants and adult rodents resulting from exposure to *S. chartarum* spores including trichothecenes, atranones, hemolysins, and proteinases and their possible synergy. Initial studies suggest that proteinase content, whereas dependent upon growth conditions, may be comparable (~twofold difference) across the two chemotypes of *S. chartarum* but that hemolysin content may be greater in the atranone-producing chemotype (Yike and Dearborn, 2004; Yike *et al.*, 2007).

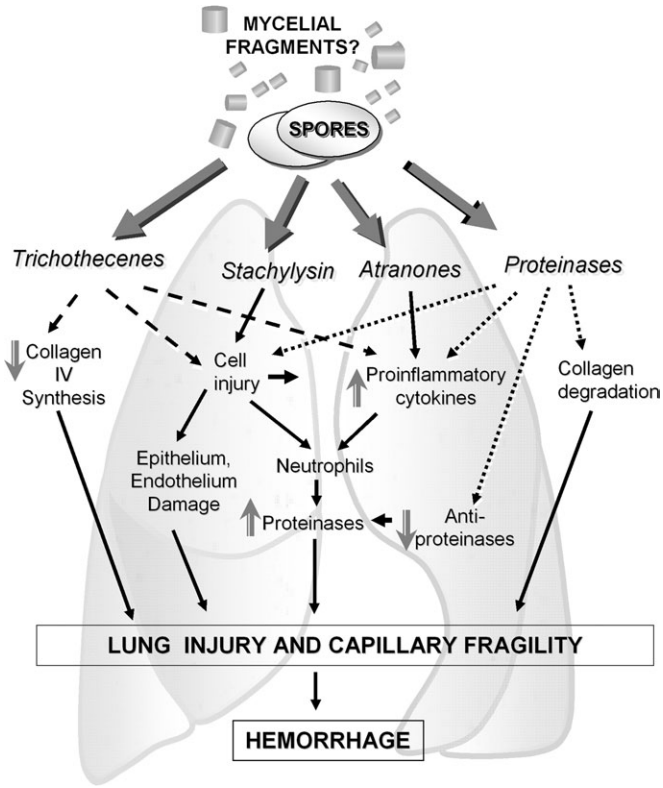

FIG. 6. Putative mediators and pathways for lung injury in rodents resulting from exposure to *S. chartarum* spores and their possible synergy. Although these mechanisms can produce injury regardless of age, fragile capillaries leading to alveolar hemorrhage are especially seen with young infant rodents who are still rapidly making new lung capillaries and have less robust defenses against injury. Adapted from Yike *et al.* (2007).

Glucans

(1 → 3)-β-D-Glucan is a cell wall component common to environmental fungi including *Stachybotrys* that has been

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linked to the development of inflammatory reactions (Beijer *et al*., 2002; Young *et al*., 2003). (1→3)-β-D-Glucan has been shown to exacerbate the inflammatory effects of dust on the upper airways of human volunteers (Bonlokke *et al*., 2006). Inhalation of (1 → 3)-β-D-glucan in humans evokes upper respiratory tract symptoms and induces cytokine production by blood monocytes (Rylander, 1999).

Dectin-1 has been suggested to be the primary receptor involved in innate recognition of fungal β-glucan. Rosenblum *et al*. (2006) observed that C57BL/6J mice are much less susceptible than BALB/c mice to the effects of intratracheal instillation with *S. chartarum* spores relative to BALF profile as well as production of chemokines and cytokines. Because C57BL/6J contain a dectin-1 splice variant that is homologous to a splice variant found in humans, it was speculated by the study's authors that human populations having this splice might exhibit decreases in both recognition of and inflammatory responses to *S. chartarum*.

Spirocyclic Drimanes

Stachybotrys produces up to 40 different spirocyclic drimanes, some in large quantities (Andersen, 2002; Nielsen *et al*., 2002). These compounds are produced in part by the terpenoid pathway, which generates two lower rings under the spiro bond, and partly by the polyketide pathway, which produces the upper part of the molecule (Jansen and de Groot, 2004). Spirocyclic drimanes have a wide spectrum of biological activities including inhibition of proteolytic enzymes, disruption of the complement system, inhibition of TNF-α release, endothelin receptor antagonism, and stimulation of plasminogen, fibrinolysis, thrombolysis, and cytotoxic and neurotoxic effects. Spirocyclic drimanes might play an as yet unidentified role in the pathophysiologic effects of the *Stachybotrys*.

Volatile Organic Compounds

Stachybotrys chartarum produces microbial volatile organic compounds that are highly dependent on isolate and culture media (Gao and Martin, 2002). However, using a sensitive mouse bioassay in which the airway reactions were measured plethysmographically, Wilkins *et al*. (1998) found that inhalation of *S. chartarum*–derived vapors cause negligible sensory irritation, bronchoconstriction, or pulmonary irritation.

EFFECT OF OTHER ENVIRONMENTAL COFACTORS

The effects of *Stachybotrys* might be potentiated by other natural and anthropogenic contaminants present in damp building indoor air including microbes, their metabolites, and chemical toxicants. Endotoxins are lipopolysaccharide (LPS)–protein complexes derived from the Gram-negative bacterial outer membrane that mediate acute inflammation during infection (Andersson *et al*., 1997). Inhalation exposure to endotoxin is well-documented in the home and workplace (Jarvholm, 1982, 1987; Jarvholm *et al*., 1982; Oxhoj *et al*., 1982; Pernis *et al*., 1961; Rylander, 1987; Salvaggio and Seabury, 1968). Instillation of LPS into the airways of laboratory rodents causes a similar inflammatory response to those observed in humans, including specific inflammatory cytokine production and neutrophil infiltration. Structural and cellular changes have been documented in the airways of laboratory rodents elicited by intranasally instilled and aerosolized LPS (Gordon and Harkema, 1994; Harkema and Hotchkiss, 1991, 1992; Steiger *et al*., 1995).

Trichothecene toxicity is potentiated by LPS, thereby causing elevated tissue injury and mortality (Tai and Pestka, 1988; Taylor *et al*., 1989, 1991; Zhou *et al*., 1999, 2000). Coexposure of mice to subtoxic doses of LPS and deoxynivalenol markedly upregulates proinflammatory cytokine expression and subsequently induces apoptosis in thymus, Peyer's patches, bone marrow, and spleen via a novel glucocorticoid-related mechanism (Islam and Pestka, 2003, 2005; Islam *et al*., 2002, 2003). Coexposure of macrophages to either LPS or irradiated *Salmonella* enhances the capacity of satratoxin to induce cytokine expression (Chung *et al*., 2003c; Mbandi and Pestka, 2006). Recently, Islam *et al*. (2007) demonstrated that roridin A–induced proinflammatory gene expression, apoptosis of OSN and inflammation in the nasal airways (acute rhinitis) was magnified by simultaneous exposure to LPS. Thus, macrocyclic trichothecene toxicity to the nasal tract might be augmented by concomitant LPS exposure.

Spores of the actinomycete *Streptomyces*, which is also present in damp buildings, can evoke significant inflammatory reactions including production of cytokines and generation of reactive nitrogen and oxygen species (Hirvonen *et al*., 1997; Jussila *et al*., 2001). Coculture of *Streptomyces californicus* with *Stachybotrys* can potentiate cytokine induction (Huttunen *et al*., 2004; Penttinen *et al*., 2005) as well as apoptotic cell death by triggering mitochondrial membrane permeabilization leading to caspase-3 activation and DNA fragmentation is potentiated by cocultivation with *S. chartarum* (Penttinen *et al*., 2007).

Environmental tobacco smoke (ETS) might also exacerbate the effects of *Stachybotrys*. ETS exposure was a significant covariant in a case–control study of Cleveland infants exhibiting pulmonary hemorrhage in which subsequent exposure of these patients to ETS resulted in rebleeding (Etzel *et al*., 1998). This relationship was tested by exposing 7-day-old rat pups to highly toxic spores or vehicle control (Yike and Dearborn, 2004). After 48 h, half the pups in each group were exposed to cigarette side stream smoke for three 15-min periods. Barometric plethysmography revealed that after smoke inhalation, the minute volume (MV) decreased in *S. chartarum*–exposed pups by 28%, whereas smoke inhalation alone did not significantly alter MV in saline-treated pups. This decrease was

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attributed to both decreased respiratory rate and increased respiratory resistance. Although these data suggest that respiratory function in *S. chartarum*–exposed rat pups is quite sensitive to acute smoke exposure, no discernable differences in inflammation or hemorrhage were observed between spore-challenged rats exposed to smoke or vehicle.

ANALYTICAL ASPECTS IN MEASURING *STACHYBOTRYS* EXPOSURE

To predict risks of exposure to *S. chartarum* or its products, the dose-dependent effects observed in animal studies must be considered in the context of actual human exposure in water-damaged dwellings and workplaces. This requires both appropriate and robust protocols for both sampling and analysis. Sampling strategies for indoor fungi *per se* can be quite complex relative to goals, hypothesis design, methods, and equipment. Although not discussed here, Portnoy *et al.* (2004) have reviewed these strategies in elegant detail and proposed investigative strategies and guidelines for appropriate sampling of indoor fungi. One notable recent advance in this area is the development of a personal sampler that uses a two-stage cyclone to collect bioaerosols into disposable micro-centrifuge tubes (Lindsley *et al.*, 2006). This fractionates aerosol particles by aerodynamic diameter and has been applied to aerosolized fungal spores.

Analytical measurement of a fungus that can (1) exist as spores or mycelia, (2) be viable or nonviable, or (3) produce a spectrum of biologically active products constitutes a challenge not encountered when measuring a single chemical toxicant. Multiple strategies have been taken for detection and measurement of *S. chartarum* or its products, each having specific advantages and disadvantages.

Microbiological

Conventional approaches for *Stachybotrys* detection in suspect air or dust samples involve culture on nonselective or selective media as well as direct microscopy. Although these methods are straightforward, they are time consuming, lack specificity, are often qualitative, yield results that can vary greatly within and between the labs, and can grossly underestimate injured or nonviable spores. During culturing, the potential exists for competing molds or bacteria to produce antibiotics that inhibit *Stachybotrys*. Because the *S. chartarum* spore *is* likely to be cleared from airway tissue of exposed individuals rapidly, these methods have questionable applicability to monitoring exposures to the organism in clinical samples.

Polymerase Chain Reaction

Real-time PCR is an extremely specific assay for *S. chartarum* that has a limit of detection of two copies of fungal genome (Black and Foarde, 2007; Haugland *et al.*, 1999; Roe *et al.*, 2001). It has been applied to environmental samples such as airborne particles, house dust, and other bulk samples (Vesper *et al.*, 2004, 2007). PCR will detect submicron fragments of the fungus only if they contain the segment of DNA recognized by the primers. In a direct comparison of parallel settled dust aliquots from visibly moldy homes in Cleveland, culturing on three different media found *S. chartarum* in only 3.5% of the homes whereas PCR detected it in 71% (Dearborn, Yang, and Vesper, unpublished data). Limitations of PCR to date are that it does not yet allow discernment of *Stachybotrys* chemotypes and it does not estimate toxin presence. Because the survival time of the organism in immunocompetent hosts is short, PCR also has limited applicability to clinical samples (upper and lower respiratory tract) taken even temporally close to exposure.

Trichothecene Bioassay

A versatile, sensitive bioassay for trichothecenes based on inhibition of luciferase mRNA translation has been described (Yike *et al.*, 1999). Because this assay is function based, responses are directly proportional to the concentrations of the most highly toxic trichothecenes. Using this approach, satratoxin concentrations ranging from 2 to 34 ng/m^3 and 54 to 330 ng/m^3 were estimated to occur in rooms of a *Stachybotrys*-contaminated home (Vesper *et al.*, 2000a). Problems with this assay relate to potential interference by incompletely removed solvents employed for sample extraction (Black *et al.*, 2006) and coextraction of contaminants capable of suppressing cell-free protein synthesis. A further limitation is that the assay is highly specific for trichothecenes and therefore not informative about other mycotoxins or products of *Stachybotrys* that do not inhibit protein synthesis.

Immunochemical Detection of Stachybotrys

A species-specific monoclonal antibody (mAb 9B4) capable of differentiating between spore preparations of *S. chartarum* and spores and mycelia of 60 other fungal species commonly found in indoor environments has recently been described (Schmechel *et al.*, 2006). Initial antigen characterization suggested that mAb 9B4 reacts with a 19- to 20-kDa protein that is expressed in spore preparations but not mycelium of *S. chartarum*. This antibody was successfully applied to confocal detection of experimentally aerosolized fungal spores of *S. chartarum* (Green *et al.*, 2006). An advantage of this assay is that it will allow detection of nonviable spores; however, a disadvantage is that it might not be applicable to detection of submicron mycelial fragments.

A sensitive competitive enzyme-linked immunosorbent assay (ELISA) was used to measure stachylysin in the conidia of 91 common indoor fungi (Van Emon *et al.*, 2003). Although five other species besides *Stachybotrys* contained stachylysin, the concentrations were at least 2000 times lower indicating

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high species specificity of the assay. Stachylysin is detectable in the sera of rats chronically exposed to low doses of *S. chartarum* as well as in pooled sera from patients exposed to *S. chartarum* indoors suggesting that stachylysin could be a potential biomarker of *S. chartarum* acute exposure. Although this assay has value for detection of fungal exposure and could be a useful adjunct in animal investigations, it is not applicable to toxin exposure because all tested *S. chartarum* isolates appear to produce stachylysin (Vesper *et al.*, 1999). In addition, the purification of stachylysin is complicated by protein aggregation and it does not appear that the polyclonal antibodies recognize a single antigen (Yike and Dearborn, personal communication).

Xu *et al.* (2007) recently isolated a 34-kDa protein produced specifically by *S. chartarum* grown on natural substrates. The protein is present at high concentrations in spores and mycelia. Polyclonal antibodies generated against this protein did not react with proteins from a taxonomically diverse group of fungi, suggesting that it could be useful as a marker for the fungus in environmental samples. These promisins antibodies have been used to detect and monitor S. chartarum spores in the luns of the mouse (Rand and Miller, 2007).

ELISAs have also been developed using *S. chartarum* fungal extracts to detect antibody responses in humans (Vojdani, 2004, 2005; Vojdani *et al.*, 2003). Although small increases in specific IgG, IgM, and IgE concentrations were reported in human sera, these data conflict with prior reports that such assays are hindered by cross-reactivity among different fungal antigens. Further concerns about this approach have been raised regarding confirmation of *Stachybotrys* exposure in positive individuals, lack of negative control sera from unexposed individuals and specific methodological problems (Page *et al.*, 2005). Commercial immunoassay kits for identifying trichothecene-specific antibodies in human blood and urine are being marketed. These protocols, however, have not been analytically validated and thus are not recommended for clinical use (http://www.bt.cdc.gov/agent/trichothecene/casedef.asp).

Immunochemical Detection of Macrocyclic Trichothecenes

Polyclonal antibodies specific for SG have been generated and employed in a specific competitive direct ELISA for measuring this and other macrocyclic toxins in *Stachybotrys* cultures (Chung *et al.*, 2003a). Brasel *et al.* (2005b) used variant of this ELISA to analyze air samples from *S. chartarum*–contaminated buildings, and found that trichothecene concentrations ranged from 10 to 1300 pg/m^3 of air. Detectable toxin concentrations were found to increase with the sampling time and short periods of air disturbance. The same group used this ELISA to analyze sera from occupants of contaminated (test samples, $n = 44$) and uncontaminated (control samples, $n = 26$) (Brasel *et al.*, 2004). Although half of the test samples were significantly different from normal human serum tested in the same manner, only one of the control samples tested positive. Although this carefully controlled study strongly suggested the presence of the toxin or its metabolite in patient samples, the exact nature of the detected substance was not determined.

Charpin-Kadouch *et al.* (2006) used this ELISA to compare macrocyclic trichothecene levels in an index group comprising 15 flooded dwellings contaminated by *S. chartarum* or *Chaetomium* and a control group comprising nine dwellings without molds on visual inspection and mold sampling. Mean levels of trichothecenes in wall, floor, and airborne dust samples from contaminated homes were 40, 22, and 0.6 ppb, respectively, and 0.3, 0.3, and 0.3 ppb, respectively, in samples from control homes. The data suggest that even though SG and other macrocyclic trichothecenes could disperse in an *S. chartarum*–contaminated dwelling they were not detectable in a single 4-h air sample.

An advantage of the competitive direct ELISA is that it is highly sensitive and can detect multiple macrocyclic trichothecenes. Similar competitive ELISAs have been widely used for screening of mycotoxins in food and agricultural commodities (Pestka, 1988). A limitation of this assay is that it does not detect other mycotoxins or products of *Stachybotrys*.

Trichothecene Adducts

Yike *et al.* (2006) recently employed exhaustive digestion, affinity column enrichment and MS analysis to discern the presence of lysine-, cysteine-, and histidine-SG adducts in the samples from patients with documented *S. chartarum* exposure (Fig. 3). Similar adducts were found in the sera of rats instilled with the spores of *S. chartarum* but not in those from control animals. These adducts were also detected in the sera from two domestic cats exposed to a moldy environment that developed pulmonary hemorrhage during anesthesia but not control cat sera (Mader *et al.*, 2007). Although the exact structures and mechanisms of the adduct formation require further study, macrocyclic trichothecenes produced by *S. chartarum* contain one or two epoxides that could mediate protein adduct formation. The possibility that this reaction might occur with blood proteins in pulmonary capillaries is supported by the observation that free SG is detectable in the blood of rat pups exposed to the *S. chartarum* spores only immediately following exposure. Because they can be detected in small samples of blood (< 0.5 ml), satratoxin–albumin adducts appear to be highly promising biomarkers of inhalation exposure to *S. chartarum*.

RESOLUTION OF THE *STACHYBOTRYS* ENIGMA

Animal studies indicate that *S. chartarum* and its products have the potential to be allergenic, inflammagenic, and cytotoxic suggesting that it indeed has unique bioactivities compared with other fungi encountered in damp buildings. An IOM expert committee concluded that although *in vitro* and

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in vivo research on *S. chartarum* suggests adverse effects in humans might be biologically plausible, there is currently inadequate evidence to establish a definitive linkage between this organism and DBRI (Institute of Medicine, 2004). Resolving the enigma of whether or not *Stachybotrys* contributes to DBRI is extraordinarily challenging given the complexity of this fungus as well as the associated sampling and analytical challenges. Because of the variability in *S. chartarum* preparations and study design differences, it is often difficult to directly compare data from different investigations. There are several critical issues that must be considered by researchers who design and conduct future *Stachybotrys* investigations using animal models. Attention to these will improve comparability.

Spores

Viability, mycotoxin type/content, and activity of fungal proteins can vary greatly among the spores of different *S. chartarum* isolates with the protein content being greatly affected by growth medium. Growth of *S. chartarum* on drywall or cellulose media yields spores with characteristics similar to those found in an indoor environment, whereas spores produced on conventional mycological media might contain higher levels of proteinases that increase their injurious effects when delivered to the respiratory tract (Yike *et al.*, 2007). Future studies should therefore include critical information about spore preparations including chemotype, toxin content, viability, and levels of proteinase activity.

Mycelial Fragments

The relative contributions of airborne spores, hyphae, and fungal fragments from *S. chartarum* to toxicity, inflammation, and allergic sensitization are not well understood. Tucker *et al.* (2007) monitored conidial dispersal in *S. chartarum* in response to low-velocity airflow using a microflow apparatus and reported that spores of this fungus were poorly adapted for dispersal by airspeeds that prevail in the indoor environment. It was speculated that even in buildings that are heavily contaminated with *S. chartarum*, only modest numbers of spores would become airborne unless contaminated surfaces are disturbed by high airspeeds or vibration. When Cho *et al.* (2005) analyzed the aerodynamic characteristics of *S. chartarum* particles released from contaminated surfaces under controlled laboratory conditions using an electrical low-pressure impactor, they found that submicron particles of *S. chartarum* can be aerosolized at 500 times greater concentrations than spores. Respiratory deposition models of human adult and infant airways suggest that submicron fragments would be deposited in 230- to 250-fold greater numbers than spores and target the lower airways, whereas spores, particularly those that are hydrated, would largely be deposited in the upper respiratory tract (Fig. 7). A recent study by Seo et al. (2007) revealed that particle release by S. Chartasum is much greater when the tunsus is grown on gypsum board on celling tiles than on an agar substcate. Brasel *et al.* (2005a) also observed submicron particles in cultures o*f S. chartarum* grown on wallboard. Therefore, further study on the size-distribution and biologic effects of *Stachybotrys* submicron particles is needed in conjunction with their measurement in water-damaged buildings.

Purified Toxins

Although investigations with spores or culture extracts are useful, studies of purified toxins are powerful and essential adjuncts that can provide insight into underlying mechanisms and tissue targets. Efficient protocols for production, radiolabeling, and analysis of these compounds would greatly facilitate toxicokinetic, disposition, and toxicogenomic studies in experimental animals. In addition, new information is needed on the profiles and environmental concentrations of trichothecenes, atranones, spirocyclic drimanes, and other secondary metabolites produced by *S. chartarum*. To date, analytical methods for *Stachybotrys* toxins have not been subjected to rigorous evaluation by assessing recoveries following spiking of diverse samples at the intra- and interlaboratory level as has been commonly done in the analysis for many foodborne mycotoxins (Pestka, 1988). Clearly, further evaluative studies are needed on both conventional and immunochemical analytical methods for determining mycotoxins in *Stachybotrys* isolates as well as prototypical environmental and clinical samples using intra- and interlaboratory approaches.

Animal Models

Species, strain, age, and gender are also important considerations in experiment design. Regarding rodents, mice appear more susceptible to inhaled *Stachybotrys* spores than rats based on the spore concentrations required to evoke pathologic effects (Flemming *et al.*, 2004; Rand *et al.*, 2003; Yike *et al.*, 2002, 2003). Although C3H/HeJ, BALB/c, and C57BL/6J all show significant dose-dependent responses to *S. chartarum* spores relative to myeloperoxidase activity, albumin, and hemoglobin levels, and neutrophil numbers in BALF, the strains are differentially sensitive with BALB/c mice being most affected and C57BL/6J mice being least affected. Furthermore, although most studies employ juvenile and young adult animals, rat pups have been used to provide particular insight on affects on human infants (Yike *et al.*, 2002, 2003). Finally, because it is widely recognized that rodent airways differ in many regards from those of humans and other primates, it will be desirable to validate novel targets and mechanisms in nonhuman primates.

Pathophysiologic Mechanisms

Allergy can cause dramatic adverse responses to even a minute concentration of an allergenic agent. *Stachybotrys chartarum* has been shown to contain IgE reactive proteins when probed with human sera. Additionally, animal studies

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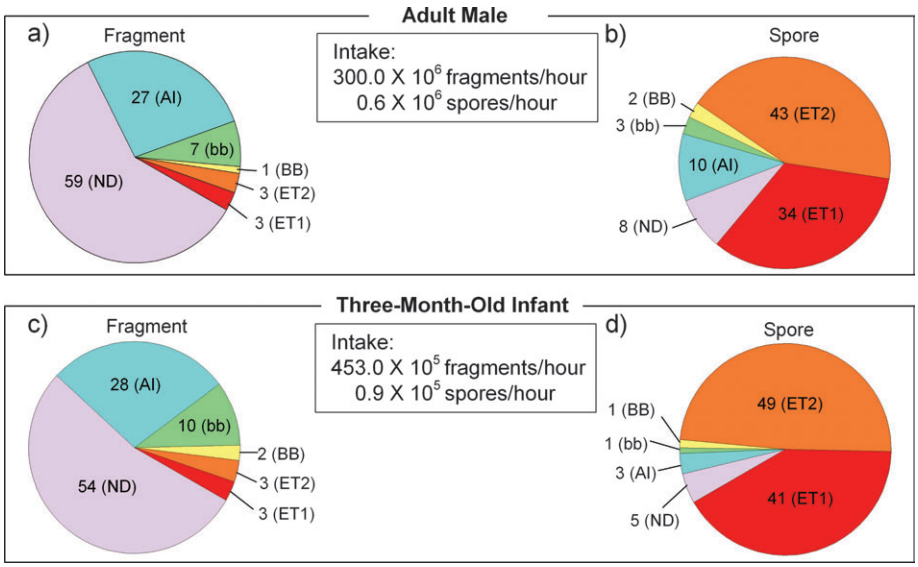

FIG. 7. Modeling deposition of *S. chartarum* fragments and spores into different regions of the respiratory tract of human adult (A, B) and infant (C, D) after 1 h of exposure. The aerodynamic characteristics of *S. chartarum* particles released from contaminated surfaces under controlled laboratory conditions were analyzed using an electrical low-pressure impactor. These data were then used to calculate total and fractional depositions of *S. chartarum* fragments and spores in an adult human male and a 3-month-old human infant. Abbreviations: ET1, anterior nasal region; ET2, main extrathoracic region; BB, bronchial region; bb, bronchiolar region; AI, alveolar interstitial region; and ND, not deposited particles. Reproduced with permission from *Atmospheric Environment* (Cho *et al*., 2005).

models suggest that *S. chartarum* has the potential to induce responses that are characteristic of human allergic asthma and rhinitis (Fig. 3). The identification of unique *S. chartarum* (or other fungal) allergens would be useful as possible biomarkers of disease etiology and/or environmental contamination. However, because spore and mycelial extracts are likely to contain different antigenic protein profiles, evaluation of all fungal components is necessary. Another critical consideration is whether macrocyclic trichothecene adducts induce untoward immune responses in a similar fashion to drugs such as penicillin (Blanca *et al*., 2005).

The observations that SG and other macrocyclic trichothecene are directly toxic to OSNs and initiate an inflammatory response in the nose (rhinitis) that extends into the brain (mild focal encephalitis) (Islam *et al*., 2006, 2007) (Figs. 4 and 5) raises new significant questions about neurodegenerative hazards associated with exposure to this fungus. Numerous studies have indicated that aging is accompanied by olfactory loss; anosmia is also seen in several neurodegenerative disorders including Alzheimer's and Parkinson's diseases (Kovacs, 2004). Future research should therefore address whether exposure to macrocyclic trichothecenes can impact olfaction and/or neurocognition.

Future work should elucidate the effects of different fungal components on multiple inflammatory pathways, cytokine networks, and cross-communications (Fig. 6). It will be important to discern how proinflammatory actions of mycotoxins and fungal proteins may change the proteolytic balance and remodeling of the extracellular matrix in order to better understand pathological conditions, including chronic inflammatory disorders (e.g., fibromyalgia, neurodegenerative disorders), with apparent and/or potential links to mold exposure. Covalent adducts made by the trichothecenes mycotoxins could conceivably play a role in chronic immune and nonimmune processes. The role of inflammation and environmental stressors in pulmonary hemorrhage needs further studies employing sensitive, noninvasive approaches such as measurements of carbon monoxide diffusing capacity.

Exposure Route

Inhalation exposure is the criterion standard approach for studying the potential adverse effects of inhaled xenobiotics. A National Research Council committee on animal models for testing interventions against bioterrorism agents has recently published helpful guidelines for designing inhalation studies, or other studies using alternative methods, for the delivery of liquid or dry powder aerosols to the respiratory tracts of laboratory animals (National Research Council, 2006). To date there have been no animal studies of *Stachybotrys* or its mycotoxins using inhalation aerosol delivery. This is most likely due in part to the high cost of conducting proper inhalation exposures, lack of adequate laboratory expertise and equipment for conducting safe and well-monitored animal exposures to a toxic aerosol, and the lack of available fungal material for generating a uniform, well-characterized, and sustainable aerosol for acute, subchronic, or chronic exposures. Therefore, alternative methods of delivery to the upper and lower respiratory tracts of laboratory rodents have been used by investigators (e.g., intracheal or intranasal instillations).

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The route employed to deliver fungal spores, extracts, or purified toxins into the airways of experimental animals greatly affects the degree to which upper and lower airways are impacted. For identification of targets and mechanisms, both intratracheal (tracheostomy, direct instillations and aspiration) and intranasal exposure, have been employed to assess effects of inhalation exposure to *S. chartarum* or its byproducts. For investigating lower airway exposure noninvasive, intratracheal delivery (Brain *et al.*, 1976) is a practical and efficient route especially for multiple exposures. Intranasal delivery of purified macrocyclic trichothecenes has been effective in demonstrating olfactory toxicity and rhinitis (Islam *et al.*, 2006, 2007). Intranasal exposure to spores also produces nasal pathology in the nose but results in variable lung responses that must be overcome by use of higher numbers of experimental animal (Yike and Dearborn, 2004).

Ultimately, the paradigms and models evolving from the aforementioned mechanism studies must be verified, albeit costly, by conventional inhalation toxicity testing of *S. chartarum* culture materials. Such testing in rodents involves aerosol generation and exposure via whole-body or nose-only chambers. Pauluhn and Thie (2007) reviewed the challenges of such approaches and noted that for short-term studies, exposure models have shifted from whole-body to nose-only systems that minimize rebreathing of atmospheres, rapidly attain equilibrium and optimize uniformity of dynamic mixing. Other key advantages to nose-only exposure chambers that might make it make attractive for *S. chartarum* testing include; (1) reduced exposure via the dermal route from preening, (2) minimal requirements for test material, (3) reduced potential for test material to react or degrade, and (4) animal welfare considerations.

Dose and Exposure Regimen

The effects of airway exposure to *S. chartarum* and its mycotoxins are dose dependent (Flemming *et al.*, 2004; Islam *et al.*, 2006; Leino *et al.*, 2003; Nikulin *et al.*, 1997; Rand *et al.*, 2006; Rao *et al.*, 2000a,b; Yike *et al.*, 2002). Although spore doses as high as 1–8 $\times$ 10^8 spores/kg bw have been employed to induce acute, frank injury, or inflammation (Yike and Dearborn, 2004), studying the effects of lower spore doses is more physiologically relevant. Many of the adverse effects can still be observed with much lower doses and the NOAEL for proinflammatory cytokine production in mice has been proposed to range from 1 to 1.5 $\times$ 10^4 spores/kg bw (Flemming *et al.*, 2004). The NOAEL and LOAEL for SG-induced nasal olfactory toxicity were 5 and 25 μg/kg bw, respectively (Islam *et al.*, 2006). Assuming a concentration of 1 pg/spore (Yike and Dearborn, 2004), these values would equate to 5–25 $\times$ 10^6 spores/kg bw. A major limitation of most *S. chartarum* spore and toxin studies to date is that most have focused on the effects of acute exposure. Animal investigations employing short-term repeated and chronic exposure to much lower doses would more closely mimic human indoor exposure and facilitate elucidation of putative pathophysiological disease.

Environmental Cofactors

DBRI is unlikely to be caused by a single agent but rather is caused by multiple factors and expressed as a spectrum of clinical symptoms. Thus, it is important to understand how *S. chartarum*'s effects might be potentiated by other natural and anthropogenic contaminants present in indoor air including microbes, their metabolites and chemical toxicants of specific interest are ETS, endotoxin, and actinomycetes.

Biomarkers

A considerable complication faced in epidemiological investigations of *Stachybotrys* is that environmental air or dust samples taken weeks or months after the onset of an adverse health event do not accurately reflect the state of that building when a putative illness was initiated or during long term intermittent chronic exposure. Protein adducts have been successfully used as exposure dosimeters for several toxic agents most notably aflatoxin B_1 (Groopman and Kensler, 2005). This mycotoxin is a potent carcinogen that is oxidized in the liver by cytochrome p450 to yield an epoxide which then reacts with the ε-amine of lysyl residues, either directly or through a spontaneously formed dialdehyde. The potential for SG and possibly other macrocyclic trichothecenes to covalently bind proteins may have similar applicability as a biomarker (Mader *et al.*, 2007; Yike *et al.*, 2006). Binding to serum albumin might contribute to the rapid disappearance of free toxin from blood in rats exposed intratracheally to the spores of highly toxic *S. chartarum* and demonstrates the biomarker potential of the adducts. The capacity to detect those adducts in blood samples of individuals and animals exposed to *S. chartarum* in comparison with control subjects suggests a high potential for this approach to provide a practical, quantitative dosimeter (Fig. 3). Because albumin is one of the most abundant serum proteins (60 mg/ml) with a half life in the human of ~20 days, it is a good candidate for a dosimeter of both acute (days) or recent (weeks to several months) mold exposure.

SUMMARY AND CONCLUSIONS

The causes of DBRI are likely to be complex and multifactorial with *Stachybotrys* possibly being an important contributing factor. Animal research provides public health and environmental scientists with useful tools for assessing the risks from airborne contaminants. To date, animal models indicate that airway exposure to *S. chartarum* (spores, extracts, bioactive components) can evoke allergic sensitization, inflammation, and cytotoxicity in the upper and lower respiratory tracts. Although earlier experiments were conducted with relatively high doses, more recent findings indicate that lower

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doses can elicit similar symptoms. Trichothecene toxicity appears to be an underlying cause of many of *S. chartarum's* adverse effects. *Stachybotrys* isolates that produce low levels of trichothecenes can also evoke injury and several investigations have convincingly demonstrated that other constituents including allergenic proteins, atranones, proteinases, hemolysins, and β-glucans might contribute to the pathophysiologic effects of this fungus. Animal studies of *Stachybotrys* have employed many different experimental designs, making results difficult to compare because of numerous variables including spore viability, mycotoxin content, inclusion of submicron-sized mycelial fragments, and the presence of fungal proteins. Well-characterized, standardized preparations of fungal spores, and mycelial fragments with properties representative of those found in indoor air are needed to elucidate mechanisms that might be contributory to adverse health effects. Development of experimental conditions that mimic chronic exposure to low doses and coexposure with other environmental factors will allow animal studies to more accurately reflect human indoor exposure. Ultimately, resolving the public health enigma of whether and how *Stachybotrys* inhalation evokes adverse health effects that contribute to DBRI will require state-of-the-art sampling/analytical methods to assess doses and timing of exposure to the fungus and its bioactive constituents as well as exploiting the use of relevant biomarkers.

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