Possible Role of Fungal Hemolysins in Sick Building Syndrome

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I. Introduction

The World Health Organization (WHO) definition of sick building syndrome (SBS) includes such symptoms in the building occupants as headache, distraction, dizziness, fatigue, watery eyes, runny or blocked or bleeding nose, dry or sore throat, and skin irritation (WHO, 1995). The suspected causes of SBS include various chemicals, toxins, and microorganisms. In this review we will focus on exposures to fungi (molds) as causes of SBS.

Identifying and quantifying the great diversity of fungi found indoors had been one of the main limitations to addressing the cause-effect relationship between fungal exposures and SBS. The recent development of quantitative PCR (QPCR) analysis of molds (US. Patent 6,387,652)

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has dramatically improved fungal speciation and quantification, resulting in a highly standardized process for describing the indoor fungal population. Does exposure to these fungi cause SBS? In this chapter, we will consider the hypothesis that fungal hemolysins may play a role in some symptoms observed in SBS.

Hemolysins are molecules that are designated as such because they have the ability to lyse red blood cells (RBCs). In this review, only proteinaceous hemolysins will be discussed, although the authors recognize that non-proteinaceous fungal molecules can have hemolytic effects (e.g., T-2 toxin causes hemolysis of red blood cells) (Segal *et al.*, 1983). For this review of fungal hemolysins, the artificial separation of macro-fungi (e.g., mushrooms) and micro-fungi (e.g., filamentous species and yeasts) will be used. Throughout this review, we will compare fungal hemolysins with bacterial hemolysins because bacterial hemolysins have been more extensively studied and may provide useful insights into the nature and activities of fungal hemolysins.

II. Biochemical Characterization of Hemolysins

There are primarily two types of hemolysins, designated alpha (α) and beta (β). Alpha hemolysins cause a partial lysis of the RBCs, resulting in a darkening of the media around a colony on sheep's blood agar (SBA). The beta hemolysins produce a complete lysis of the RBCs, resulting in a clearing around the colony growing on SBA. First we will review what is known about the biochemistry of hemolysins.

A. Hemolysins Produced by Macro-Fungi

The first proteinaceous, macro-fungal hemolysin was named *phallin* (Kobert, 1891). It is now called *phallolysin* and is produced by *Amanita phalloides* (Wieland, 1986). The mushroom *A. rubescens* produces the hemolysin rubescenslysin (Wieland, 1986). These proteins lyse RBCs and are unstable in acids and temperatures over 65 °C. The mushrooms *Pleurotus ostreatus* and *Agrocybe aergerita* produce the hemolysins ostreolysin and aegerolysin, respectively (Berne *et al.*, 2002). Ostreolysin has a molecular weight (MW) around 17 kDa under reducing conditions, but under non-reducing conditions, 17 and 16 kDa bands were identified. Aegerolysin has a single band of 17 kDa under reducing and a 16 kDa band under non-reducing conditions. Wang *et al.* (2002) tested 14 different mushrooms for hemolysins and found that most produced these kinds of proteins. However, none of these compounds were well characterized beyond suggesting that their MWs were greater than 10 kDa (based on dialysis).

B. Hemolysins Produced by Micro-Fungi

1. *Yeasts*

Salvin (1951) reported that hemolysins were produced by the yeast-like phases of *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Candida albicans*, and *Cryptococcus neoformans*. More recently, *C. albicans* was shown to produce a β-hemolytic agent, but it was not characterized (Manns *et al.*, 1994). In a more comprehensive study, Luo *et al.* (2001) tested the hemolytic activity of 14 *Candida* species. *C. albicans*, *C. dubliensis*, *C. kefyr*, *C. krusei*, *C. zeylanoides*, *C. glabrata*, *C. tropicalis*, and *C. lusitaniae* all produced alpha hemolysis at 24 h, which led to beta hemolysis at 48 h. *Candida famata*, *C. guilliermondii*, *C. rugosa*, and *C. utilis* produced only alpha hemolysis, and *C. parapsilosis* and *C. pelliculosa* demonstrated no hemolytic activity.

2. *Filamentous Fungi*

Henrici (1939) partially purified a hemolytic agent from ground mycelia of the filamentous fungus *Aspergillus fumigatus*. Asp-hemolysin was first fully purified in 1977 (Yokota *et al.*, 1977) and was described as having a MW of 30 kDa with an acidic isoelectric point (pI 4.0). Much later, cloning and sequencing allowed the actual MW to be determined to be 14.3 kDa, containing 131 amino acids of which three are cysteine (Ebina *et al.*, 1994). Asp-hemolysin is a secretory glycoprotein that binds to a specific receptor, the low-density lipoprotein receptor (L-DLR) (Fukuchi *et al.*, 1998) (e.g., in arterial walls) (Ebina *et al.*, 1983). However, there was little interest in examining whether other filamentous fungi produced hemolysins.

Not until 2001 was Stachylysin™ recognized as a proteinaceous hemolytic agent produced by the filamentous fungus *Stachybotrys chartarum* (Vesper *et al.*, 1999, 2001). The monomeric form of stachylysin has a MW of 11.9 kDa, as determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF-MS), with 114 amino acids, two of which are cysteine (Vesper *et al.*, 2001). More recently, chrysolysin was purified from *Penicillium chrysogenum* (Donohue *et al.*, 2004). Monmeric chrysolysin has a MW of 2 kDa, with one cysteine, and it has a pI of 4.9. These results led us to survey other filamentous fungi for hemolysin producers.

We found that hemolysins are produced by many of the common indoor fungi (Van Emon *et al.*, 2003). In this study, 90 common indoor fungi (Table I) were grown on SBA. All species tested germinated and grew on SBA at 23 °C. However, only seven species secreted a hemolysin at 23 °C. But if those same SBA plates, with growing fungi,

TABLE I

MICRO-FUNGAL HEMOLYSIN SECRETION AT DIFFERENT TIMES AND TEMPERATURES

	Incubation temperature						
	23 °C		23 °C to 37 °C			37 °C	
Fungal species[a]	Growth day 5	Hemol. day 5	Hemol. day 1	Hemol. day 2	Hemol. day 3	Growth day 5	Hemol. day 5
Absidia corymbifera	+++[b]					+++	
Acremonium strictum	+		*[c]	*	*	no	
Alternaria alternata	++					+	
Aspergillus auricomus	++		*	*	*	no	
Aspergillus caespitosus	+++		*	*	**	+	
Aspergillus candidus	+		*	*	*	no	
Aspergillus carbonarius	++		*	*	*	+	
Aspergillus cervinus	+					no	
Aspergillus clavatus	++		*	*	**	+	
Aspergillus flavipes	++		*	*	**	+	**
Aspergillus flavus	++		*	*	*	+++	***
Aspergillus fumigatus	++					+++	***
Aspergillus niveus	++		*	*	*	++	**
Aspergillus niger	+++	**	**	**	***	+++	***
Aspergillus ochraceus	++			*	*	+	*
Aspergillus paradoxus	++		*	*	*	no	
Aspergillus parasiticus	++					+++	**
Aspergillus puniceus	++		*	*	*	no	
Aspergillus restrictus	+					no	
Aspergillus sclerotiorum	++					+	**

Aspergillus sydowii	++	*	*	*	*	++	*
Aspergillus tamarii	+++		*	*	**	+++	
Aspergillus terreus	++					+++	**
Aspergillus unguis	++		*	*	*	++	**
Aspergillus ustus	++		*	**	**	+	
Aspergillus versicolor	+		*	*	**	+	**
Aspergillus wentii	++	*	*	**	***	no	
Aureobasidium pullulans	+			*	*	no	
Chaetomium globosum	+++					++	
Cladosporium cladosporioides I	+		*	*	*	no	
Cladosporium cladosporioides II	+	*	*	*	*	no	
Cladosporium herbarum	+		*	*	*	no	
Cladosporium sphaerospermum	+		*	*	**	no	
Emericella nidulans	++	*	*	*	*	+++	**
Emericella variecolor	++		*	*	*	+	*
Epicoccum nigrum	++		**	**	**	no	
Eurotium chevalieri	+		*	*	*	+	
Memnoniella echinata	++		*	*	*	+	**
Myrothecium verrucaria	++		*	*	*	+++	**
Mucor racemosus	+++					no	
Paecilomyces lilacinus	++		*	*	*	+	*
Paecilomyces varioti	+		*	*	*	++	**
Penicillium aethiopicum	++			*	*	+	**
Penicillium atramentosum	++		**	**	**	no	
Penicillium aurantiogriseum	++		*	*	*	no	
Penicillium brevicompactum	++	*	*	*	*	no	

(*continued*)

TABLE I (*Continued*)

Fungal species[a]	Incubation temperature						
	23 °C		23 °C to 37 °C			37 °C	
	Growth day 5	Hemol. day 5	Hemol. day 1	Hemol. day 2	Hemol. day 3	Growth day 5	Hemol. day 5
Penicillium canescens	+		*	**	**	+	*
Penicillium chrysogeum	++		*	*	**	+	*
Penicillium citreonigrum	+		*	*	*	no	
Penicillium citrinum	++		*	*	*	+	*
Penicillium coprophilum	++					no	
Penicillium corylophilum	+		*	*	*	no	
Penicillium crustosum	++		**	**	**	no	
Penicillium decumbens	+		*	*	*	+	*
Penicillium digitatum	+					no	
Penicillium expansum	++		*	*	**	no	
Penicillium fellutanum	+		*	*	*	+	*
Penicillium glandicola	++		*	*	*	no	
Penicillium griseofulvum	+		*	*	*	+	*
Penicillium implicatum	+					no	
Penicillium islandicum	+		**	**	**	+	**
Penicillium italicum	+					no	
Penicillium janthinellum	++		*	*	*	++	*
Penicillium lividum	+		*	*	*	no	
Penicillium melinii	+		*	*	*	no	
Penicillium miczynskii	++		*	*	*	no	
Penicillium olsonii	++		*	*	**	+	
Penicillium oxalicum	++		*	*	*	+++	*

Penicillium purpurogenum	+					+	
Penicillium raistrickii	++		*	*	**	no	
Penicillium restrictum	+		*	*	*	+	**
Penicillium roquefortii	++		*	*	*	no	
Penicillium sclerotiorum	++		*	*	*	no	
Penicillium simplicissimum	++					+	*
Penicillium spinulosum	++		*	*	*	no	
Penicillium variabile	+		*	*	*	no	
Penicillium verrucosum	++					no	
Penicillium waksmanii	++					no	
Rhizopus stolonifer	+++					no	
Scopulariopsis brevicaulis	++		*	*	*	++	*
Scopulariopsis brumptii	+		*	*	*	+	
Scopulariopsis chartarum	+		*	*	*	no	
Stachybotrys chartarum	++	*	***	***	***	++	***
Trichoderma asperellum	++					++	
Trichoderma harzianum	+++					+	
Trichoderma longibrachiatum	+++		***	***	***	+++	***
Trichoderma viride	+++					no	
Ulocladium atrum	++					+	*
Ulocladium botrytis	++					+	*
Ulocladium chartarum	++					+	
Wallemia sebi	+					no	

[a]The Culture Collection source of each of these species can be found in Van Emon *et al.*, 2003.
[b]One plus sign indicates a small amount of growth after 5 days, two indicate moderate growth, and three indicate abundant growth.
[c]One star indicates a small amount of hemolytic activity, two indicate moderate activity, and three indicate abundant hemolytic activity.

were transferred to 37 °C, then 65 species secreted hemolysins. On the other hand, only 50 of the 90 fungi were able to germinate and grow on SBA at 37 °C (Table I). Of the 50 that germinated, only 36 showed hemolytic activity (Table I).

We are in the process of biochemically characterizing these other micro-fungal hemolysins. So far, all of these hemolysins demonstrate a characteristic response on SBA—that is, there is initial darkening of the SBA medium beyond the colony. This darkened area turns bluish green and then clears over 24 to 48 h (Van Emon *et al.*, 2003; Vesper *et al.*, 2001). (This is similar to the slow reaction described by Luo *et al.* [2001] for the *Candida* hemolysins.)

C. Bacterial Hemolysins

Bacterial hemolysins are a fairly heterogenous group of proteins, but they have been classified in many cases into three ''families'' of toxins. The beta-sheet-structured (BSS) family is so named because of the abundance of a β-sheet structure in the monomer. An example is alpha-toxin, produced by *Staphylococcus aureus*. Alpha-toxin has a molecular weight of 33.4 kDa made up of 297 amino acids but no cysteine. It produces oligomers that are heptamers and forms pores of 6–10 Å.

The members of the cholesterol-binding toxin family (C-BT) are thiol-activated and bind to cholesterol in membranes. An example is streptolysin-O, which has a molecular weight of 61.5 kDa and is made up of 538 amino acids with one cysteine. It forms oligomers of 25 to 80 monomers and produces large pores, up to 30 nm in diameter.

The repeats-in-toxin (RTX) family is so named because the C-terminal half of the molecule contains multiple repeats of a peptide sequence. These hemolysins are usually produced by Gram-negative bacteria and range in size from 102 to 178 kDa (Stanley *et al.*, 1998). For example, *Escherichia coli* α-hemolysin has a MW of 110 kDa with 1024 amino acids but no cysteine. Members of this family may or may not form oligomers. The pores produced are 10–15 Å in diameter. A fairly comprehensive review of the bacterial hemolysins is available (Lahiri, 2000).

D. Comparison Between Fungal and Bacterial Hemolysins

What little is known about the biochemistry of fungal hemolysins is summarized in Table II. We don't know enough yet to place the fungal hemolysins in any of the bacterial hemolysin families. In fact, at this time, it appears that fungal hemolysins are generally composed of smaller molecular weight monomers than most bacterial

TABLE II

SOME FUNGAL HEMOLYSINS, THEIR SOURCE, MOLECULAR WEIGHT (MW), ISOLECTRIC POINT (pI), AND REFERENCE

Hemolysin	Fungus	MW (kDa) monomer	pI	Reference
Aegerolysin	*Agrocybe aegerita*	16	4.85	Berne *et al.*, 2002
Asp-hemolysin	*Aspergillus fumigatus*	14.3	4.0	Ebina *et al.*, 1994
Canditoxin	*Candida albicans*	?	Acidic	Salvin, 1951
Chrysolysin	*Penicillium chrysogenum*	2	4.9	Donohue *et al.*, 2004
Flammutoxin	*Flammulina velutipes*	32	5.4	Bernheimer and Oppenheim, 1987
Ostreolysin	*Pleurotus ostreatus*	16	5.0	Berne *et al.*, 2002
Phalloyysin	*Amanita phalloides*	34	7.0, 7.6, 8.1	Faulstich *et al.*, 1983
Stachylysin	*Stachybotrys chartarum*	11.9	3.5	Vesper *et al.*, 2001

hemolysins. They usually have more acidic isoelectric points and contain more cysteine amino acids than bacterial hemolysins. Fungal hemolysins are remarkably slow acting as compared with bacterial hemolysins. However, like most bacterial hemolysins, fungal hemolysins are aggregating proteins that create pores in membranes.

III. Physiological Actions of Hemolysins as Cytolysins

Hemolysins might be described more accurately as *cytolysins*, because they lyse many cells besides RBCs. The physiological response to hemolysins depends on the type(s) of cell(s) affected.

A. CYTOLYTIC ACTIVITY OF MACRO-FUNGAL HEMOLYSINS

1. *Rubescenslysin*

The mushroom hemolysin, rubescenslysin, caused the lysis of RBCs, as expected, but leukocytes were even more susceptible to lysis than RBCs (Odenthal *et al.*, 1982). This hemolysin's broader cytolytic activity had other effects. Skeletal muscle exposed to rubescenslysin showed a loss of direct and indirect excitability, and rubescenslysin also caused membrane damage in the renal parenchyma (Odenthal *et al.*, 1982). Dosing of experimental animals resulted in vascular permeability leading to cardiotoxicity, seizures, and ultimately hemorrhagic, pulmonary edema and death (Odenthal *et al.*, 1982; Seeger *et al.*, 1981).

2. *Phallolysin*

Phallolysin destroys leukocytes as well as RBCs (Faulstich *et al.*, 1974) and is the most potent toxin produced by the mushroom *Amanita phalloides*. If injected intraperitoneally (IP), it had an LD_{50} of 40 μg/Kg in rabbits and 50 μg/Kg in rats (Faulstich *et al.*, 1983). However, if it is consumed, phallolysin is destroyed by the stomach acids. Therefore it does not contribute to human ingestion poisonings (Faulstich *et al.*, 1974, 1983).

3. *Flammutoxin*

The hemolysin flammutoxin was isolated from the edible mushroom *Flammulina velutipes*. When injected IP into mice, it caused an immediate writhing reaction (Lin *et al.*, 1975). It is a strong cardiotoxic protein and results in respiratory failure and bleeding in the lungs (Lin *et al.*, 1974). These observations indicate that this hemolysin caused significant membrane damage, similar to the other mushroom hemolysins.

B. Cytolytic Activity of Micro-Fungal Hemolysins

1. *Asp-hemolysin*

The broad cytolytic activity of asp-hemolysin has been the best documented of the micro-fungal hemolysins. A dose-dependent increase in cytotoxicty occurred in cultured mouse peritoneal macrophages exposed to asp-hemolysin (Kumagai *et al.*, 1999). Also, when human umbilical vein endothelial cells (HUVECs) were exposed to asp-hemolysin at 100 μg/ml, the viability of the HUVECs was reduced by 50% after only 1 hr of exposure. These culture-based results may explain some of the health effects observed when experimental animals were exposed to asp-hemolysin.

Inoculation of viable spores of *A. fumigatus*, together with asp-hemolysin, promoted aspergillosis infections in mice (Ebina *et al.*, 1982). These mice developed granulomatous lesions most commonly in the lung and liver, with fewer in the spleen, kidney, and intestine (Iwata *et al.*, 1962). Injections (IP) of the purified asp-hemolysin produced hemorrhagic lesions in experimental animals (Sakaguchi and Yokota, 1972) and an LD_{50} in mice of 750 μg/Kg (Sakaguchi *et al.*, 1975).

2. *Stachylysin*

Stachylysin injected into the earthworm *Lumbricus terrestis* resulted in the leakage of hemoglobin from the animal's vascular system (Vesper and Vesper, 2002). We showed that stachylysin caused pores in sheep

RBC membranes (Vesper *et al.*, 2001), but *L. terrestis* has no oxygen-carrying cells and a closed vascular system. The release of erythrocruorin hemoglobin from *L. terrestis* and the development of aneurism-like structures suggested that stachylysin caused cytolytic vascular leakage, resulting in an LD_{50} of 1100 μg/Kg in *L. terrestis* (Vesper and Vesper, 2002).

C. Cytolytic Activity of Bacterial Hemolysins

Hemolysins have been isolated and purified from many bacterial pathogens, and they are generally important virulence factors with broad cytolytic activities (Baker and Edwards, 1995; Bhakdi and Tranum-Jensen, 1991; Bhakdi *et al.*, 1990, 1996; Cavalieri *et al.*, 1984; Feldman *et al.*, 1990; Grimminger *et al.*, 1991; Johnson *et al.*, 1985; O'Reilly *et al.*, 1986; Ou *et al.*, 1988; Van Der Vijer *et al.*, 1975). The range of cytolytic and other effects associated with different bacterial hemolysins is summarized in Table III, but this is by no means an exhaustive list of either bacterial hemolysins or their effects. Additional information can be obtained in various reviews (Bhakdi *et al.*, 1996; Lahiri, 2000).

IV. Potential Roles of Micro-Fungal Hemolysins/Cytolysins in SBS

It is our hypothesis that some SBS symptoms are described as flu- and/or cold-like because some fungal hemolysins activate the same human inflammatory system (histamine and cytokine producing cells) as the rhino- and influenza viruses. In addition, other symptoms such as headache, dizziness, and bleeding may be the manifestations of some fungal hemolysin's affect on vascular tissues. Finally, we propose that the major mode of exposure is colonization by fungi of the human airway, which leads to the release of hemolysins (and other toxins) into the host.

A. Symptoms of SBS and Possible Connection With Fungal Hemolysin Exposure

The World Health Organization (WHO, 1995) defined SBS as the manifestation of at least one symptom from the following groups:

- General symptoms, such as headache, dizziness, distraction, fatigue
- Eye irritation, such as redness, watering, itching, dryness, swollen eyelids

TABLE III

EXAMPLES OF BACTERIAL HEMOLYSINS AND THE EFFECTS THEY HAVE ON CELLS, ANIMALS, OR HUMANS

Hemolysin/source	Effects	Reference
1. Adenylate Cyclase Toxin		
Bordetella pertussis	Suppression of platelet aggregation and prolongs bleeding.	Iwaki *et al.*, 1999
2. Aerolysin		
Aeromonas hydrophila	Histamine release from mast cells.	Scheffer *et al.*, 1988
3. α-Toxin		
Staphylococcus aureus	Apoptosis of T-lymphocytes;	Jonas *et al.*, 1994
	Elevation of pulmonary arterial pressure;	Walmrath *et al.*, 1993
	IL-6 induced in mice;	Onogawa *et al.*, 2002
	IL-8 induced in alveolar epithelial cells.	Rose *et al.*, 2002
4. β-Hemolysin		
Group B *Streptococcus*	IL-8 induction;	Doran *et al.*, 2002
	Cytolytic injury to human alveolar cells and pulmonary capillary endothelial cells leading to hemorrhaging;	Gibson *et al.*, 1999
	Brain invasion by breaking down blood brain barrier;	Nizet *et al.*, 1997b
	IL-6 increase; joint injury and arthritis.	Puliti *et al.*, 2000
5. α-Hemolysin		
Escherichia coli	Microcirculatory abnormalities;	Mayer *et al.*, 1999
	Release of IL-1B and IL-6;	Bhakdi *et al.*, 1996
	IL-6 and IL-8 increase;	Jantausch *et al.*, 2000
	Hemorrhaging;	Stanley *et al.*, 1998
	Vasoconstrictor and thromboxane generation.	Walmrath *et al.*, 1994
6. Pneumolysin		
Streptococcus pneumoniae	Release of histamine from mast cells;	Howell and Gomperts, 1987
	IL-6 induced in mouse lung;	Rijneveld *et al.*, 2002
	Increase in IL-8 but not TNF-α;	Cockeran *et al.*, 2002
	Ciliary slowing and epithelial disruption.	Feldman *et al.*, 1990
7. Streptolysin-O		
Streptococcus pyogenes	Release of IL-6 and IL-8 from keratinocytes and endothelial cells.	Mitsui *et al.*, 2002

- Irritated, runny or blocked nose (sometimes described as congestion, bleeding nose, itchy, dry, or blocked nose)
- Dry or sore throat, sometimes described as upper airway irritation or difficulty with swallowing
- Dryness, itching, or irritation of skin, occasionally with rash.

1. *Symptoms Associated with Inflammatory Response*

a. Histamine-Related Responses. The symptoms of SBS are often described as flu- and/or cold-like, including watery eyes, runny nose, and irritations (Harrison *et al.*, 1992). These are common manifestations of conditions in which mast cells in the nose, eyes, throat, and lungs are stimulated to release histamines. These histamines attach to nearby blood vessels, causing them to swell and secrete more fluid than usual, resulting in watery eyes and runny nose. Histamines can also irritate nearby nerve endings, causing itching. Do hemolysins cause histamine release?

Bacterial hemolysins, produced by strains of *E. coli*, caused the induction of histamine release from rat peritoneal mast cells and human basophilic granulocytes (Gross-Weege *et al.*, 1988). Similarly, *Aeromonas hydrophila, Serratia marcescens*, and *Listeria monocytogenes* hemolysins were shown to induce histamine release from rat mast cells in a dose-dependent manner (Scheffer *et al.*, 1988). Staphylococcal hemolysin and *Pseudomonas aeruginosa* hemolysin caused release of histamine from rat mast cells (Bergmann *et al.*, 1989; Prevost *et al.*, 1998). So bacterial hemolysins cause histamine release but do fungal hemolysins?

The induction of histamine release by fungal hemolysins has not been as extensively studied. Phallolysin and rubescenslysin caused disruption of rat mast cells and rapid degranulation (Seeger and Bunsen, 1980). Asp-hemolysin caused anaphylactic shock in mice (Iwata *et al.*, 1962), and flammutoxin caused the release of histamines, resulting in symptoms that were treatable with antihistamines (Lin *et al.*, 1975). Thus, bacterial and fungal hemolysins can stimulate histamine release.

b. Cytokine-Related Responses. Experimental exposures of humans to either the cold or flu virus result in the induction of cytokines leading to the typical systemic symptoms of these diseases (Hayden *et al.*, 1998; Zhu *et al.*, 1996). The cytokines, especially interleukin (IL)-6 and also interferon (INF)-α followed by tumor necrosis factor (TNF)-α and IL-8, are responsible for the virus-induced symptoms

associated with colds and flu (Hayden *et al.*, 1998; Zhu *et al.*, 1996). Do hemolytic proteins also induce these same cytokines?

The cytokines induced by both bacterial and fungal hemolysins have been measured in the tissues and cells of many different animals, including humans. For example, Staphylococcal α-toxin induced over-secretion of IL-1α and IL-6 in cultured macrophages (Onogawa, 2002) and IL-8 in human alveolar epithelial cells (Rose *et al.*, 2002). Group B streptococcal β-hemolysin induced IL-8 (Doran *et al.*, 2002). Pneumolysin introduced into lungs of mice caused a dose-dependent increase in IL-6 (Rijneveld *et al.*, 2002), and in human neutrophils, an increase in IL-8 (Cockeren *et al.*, 2002).

Cytokines were induced by the fungal hemolysin asp-hemolysin, which caused an increase in TNF-α, IL-1β, IL-6, and IL-8 in human umbilical vein endothelial cells (Kumagai *et al.*, 2001). Mouse peritoneal macrophages secreted TNF-α and IL-1α after exposure to asp-hemolysin (Kumagai *et al.*, 1999). Thus the same kinds of cytokines, especially IL-6, are induced by many bacterial and fungal hemolysins as are induced by the cold or flu viruses. This suggests that some of the SBS symptoms might be caused by fungal hemolysin exposures leading to cytokine inductions.

2. *Symptoms Associated with Vascular Tissue Damage*

The SBS symptoms of ''headache, dizziness, and nose bleeds'' may be associated with impairment of vascular tissues. There are many examples of bacterial hemolysins that damage vascular tissues (Table III). For example, alpha toxin, produced by *S. aureus*, caused increases in pulmonary arterial pressure (Walmrath *et al.*, 1993) and α-hemolysin, produced by *E. coli*, caused vascular leakage, micro-circulatory abnormalities, and vasoconstriction (Ermert *et al.*, 1992; Mayer *et al.*, 1999). Group B streptococcal β-hemolysin caused a breakdown in the pulmonary capillary endothelial cells that resulted in pulmonary hemorrhaging, which can lead to death in infants (Nizet *et al.*, 1996, 1997a). These kinds of vascular changes affect blood pressure and vascular integrity that can lead to headaches, dizziness, and bleeding.

Nosebleeds, and by extension, idiopathic pulmonary hemosiderosis, reported in Cleveland (Dearborn, 1997; Dearborn *et al.*, 1999; Etzel *et al.*, 1998) and other cities (Elidemir *et al.*, 1999; Flappan *et al.*, 1999), may also be manifestations of hemolysin damage to vascular tissue. The macro-fungal hemolysins rubescenslysin (Seeger *et al.*, 1981) and flammutoxin (Lin *et al.*, 1974) caused pulmonary bleeding in experimental animals. Pure asp-hemolysin is cytotoxic for vascular endothelial cells (Kumagai *et al.*, 2001), leading to hemorrhagic lesions

in animals (Sakaguchi and Yokota 1972). Exposure to *S. chartarum* has been shown to cause hemorrhaging in animals (Forgacs, 1972; Sarkisov and Orshanskaiya, 1944) and agricultural workers (Hintikka, 1978; Rylander,1994; Sorenson and Lewis, 1996). The hemolysin stachylysin caused hemorrhaging in an animal model (Vesper and Vesper, 2002). Thus there are a number of cases where fungal hemolysins cause damage to or destruction of vascular tissues.

B. Mode of Exposure

There are several possible modes of exposure of humans to fungi. At this time, direct inhalation of spores is considered the most likely. Once inhaled, larger fungal conidia are primarily retained in the nasal and sinus structures. Smaller conidia enter the tracheae and lungs. Because few spores are detected in the air, many believe that any toxins or other products in these few spores would not be adequate to cause a health problem. Is there another possibility?

Ponikau *et al.* (1999) have demonstrated that more than 60 different species of fungi were cultured from nasal secretions. Many of these same fungi have been demonstrated to produce hemolysins (Table II). It seems likely that these colonizing fungi would release hemolysins (and other toxins) into their host. We found that actively growing *S. chartarum* mycelia produce about 10 to 100 times more stachylysin per mg wet weight than the conidia of the same strain (Van Emon *et al.*, 2003). Does the release of hemolysins occur?

Asp-hemolysin was detected in mouse kidney and cerebrum 2 days after introduction of the *A. fumigatus* conidia (Ebina *et al.*, 1982). Stachylysin was detected by immunochemical localization around *S. chartarum* conidia in mouse lung sections, 24 h (even more at 72 h) after the lungs had been instilled with *S. chartarum* conidia (Gregory *et al.*, 2003). Stachylysin was also measured in sera of rats and humans exposed to *S. chartarum* (Van Emon *et al.*, 2003). Thus, fungal hemolysins were making their way into the bloodstream of animals and humans by some mode of exposure. We believe that colonizing fungal mycelia may be a more significant source than inhaled spores.

V. Why Do Fungi Make Hemolysins?

One of the main advantages for microorganisms producing hemolysins is the acquisition of iron that is required for their growth. There is actually very little free iron in mammals (Bullen, 1981). Bacterial hemolysins are able to lyse RBCs and thus release the iron they contain. In

fact, most infectious bacteria use this strategy to obtain iron. In many cases, the ability of pathogenic microorganisms to acquire iron from a mammalian host by producing a hemolysin has been shown to be of critical importance in establishing infections (Payne and Finkelstein, 1978). In addition to obtaining iron, many bacterial hemolysins target host immune function cells (e.g., phagocytic leukocytes, which increases a microorganism's chance of survival in a mammalian host). These strategies may also work for invasive fungal infections such as *C. albicans* and *A. fumigatus*. But why do non-infectious fungi produce hemolysins?

The simple explanation is that hemolysins must provide some advantage for survival in their environment. For example, some mushroom hemolysins have been shown to be produced only at the initiation of fruiting body development (Berne *et al.*, 2002). Wang *et al.* (2002) demonstrated that numerous mushrooms produce proteins, including hemolysins, that have insecticidal activity. The production of hemolysins by *Pleurotus* and *Agrocybe* at the time of fruiting might provide protection from insects. Perhaps hemolysins secreted at ambient temperatures by microfungi are able to destroy grazing amoebae or other predators. But actually very few common indoor air fungi secrete hemolysins at ambient temperature (Van Emon *et al.*, 2003). So it is difficult to explain why so many micro-fungi secrete hemolysins only when the temperature is raised near human body temperature.

We suggest that the primary role of micro-fungal hemolysins may be assisting the colonization of the human (or mammalian) airway (Yike *et al.*, 2003). Most people now spend more than 90% of their life indoors. As we continue to co-habitate with fungi, we may well be selecting for fungi that can not only live with us but also in us. For example, *Penicillium chrysogenum* is the most common *Penicillium* species found indoors (Summerbell *et al.*, 1992). We now know that what has been called *Penicillium chrysogenum* is made up of a number of genetically distinct species (Scott, 2001). However, regardless of the genetic species of *P. chrysogenum*, those that grow at 37 °C on SBA produce the hemolysin chrysolysin, but those that don't grow, don't produce chrysolysin (Donohue *et al.*, 2004). Further studies of the population diversity in fungal hemolysin secretion may lead to a better understanding of SBS.

VI. Application of Fungal Hemolysins in the Analysis of SBS

To better understand the role of exposures to indoor fungi, such as *S. chartarum*, in human health, quantifiable biomarkers of exposure must be developed. A biomarker should be specific to a given fungus

and yet occur in all strains of the fungus. It should also be easily measured in bodily fluids as well as environmental samples.

The low molecular mycotoxins have been considered as possible biomarkers. For example, the trichothecenes produced by *S. chartarum* have been implicated as a cause of some ill health effects in animals (Eppley and Bailey, 1973; Forgacs, 1965), but these toxins are difficult to measure because of their low concentrations in environmental samples and because of their rapid metabolism and elimination from the body (Ueno, 1983). Furthermore, not all strains of *S. chartarum* produce these mycotoxins (Jarvis *et al.*, 1998). An ELISA test for roridin-trichothecene IgG failed to distinguish between individuals exposed and not exposed to *S. chartarum* (Trout *et al.*, 2002).

As an alternative, we propose measuring the proteinaceous fungal hemolysins to quantify fungal exposures. For example, stachylysin is produced by all strains of *S. chartarum* (tested so far) and is essentially specific to *S. chartarum* (Van Emon *et al.*, 2003). Since stachylysin is highly immunogenic, it was easily measured by ELISA in rats and human serum (but was not detected in the serum of controls) and in environmental samples like dust (Van Emon *et al.*, 2003). We are in the process of developing similar assays for the other hemolytic producing fungi.

VII. Conclusions

Although this chapter has discussed exclusively fungal hemolysins in SBS, other microorganisms and/or other products might be just as important or even more important in causing the symptoms of SBS. In fact, it seems likely that there are interactions of many agents and toxins in the symptomatology of SBS. However, some points about fungal hemolysins that are relevant to SBS include:

- Fungal and bacterial hemolysins have much in common;
- Fungal hemolysins are commonly produced;
- Fungal hemolysins could cause some symptoms of SBS;
- Fungal hemolysins might be useful as biomarkers of exposure to indoor fungi, since they can be measured in bodily fluids and environmental samples.

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