AIRBORNE FUNGAL FRAGMENTS AND ALLERGENICITY

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Exposure to fungi, particularly in water damaged indoor environments has been thought to exacerbate a number of adverse health effects including subjective symptoms such as fatigue, cognitive difficulties and problems with memory to more definable diseases such as allergy, asthma and hypersensitivity pneumonitis. Understanding the role of fungal exposure in these environments has been limited by methodological difficulties in enumerating and identifying fungi in environmental air samples. Consequently data on personal exposure and sensitization to fungal allergens has been restricted to the spores of a few select and easily identifiable species. The contribution of airborne spores, hyphae and fungal fragments of other genera to exposure and allergic sensitization are poorly characterized. There is increased interest in the role of aerosolized fungal fragments following reports that the combination of hyphal fragments and spore counts improved the association with asthma severity [1]. Such fragments are categorized as either sub-micronic particles or larger fungal fragments. In vitro studies have shown that sub-micronic particles of several fungal species are aerosolized in much higher concentrations (300-500 times) compared to spores [2], and that respiratory deposition models suggest that these particles of Stachybotrys chartarum may be deposited 230-250 fold higher than spores [3]. The practical implications of these models are yet to be determined for actual human exposures.

We have developed novel immunodetection techniques to determine the extent to which larger fungal fragments, including hyphae and fractured conidia function as aeroallergen sources. These were based on the Halogen Immunoassay (HIA), an immunostaining technique that detects membrane-bound antigens derived from collected airborne particles >2 μm with human serum IgE [4]. Our studies demonstrate that the numbers of total airborne hyphae were often significantly higher in concentration than conidia of individual allergenic genera [5]. Approximately 25% of all hyphal fragments expressed detectable allergen and the resultant localization of IgE immunostaining was heterogeneous among the hyphae. Furthermore, conidia of ten genera that were previously uncharacterized could be identified as sources of allergens. These findings highlight the contribution of larger fungal fragments as aeroallergen sources and present a new paradigm of fungal exposure [5].

Direct evidence of the associations between fungal fragments and building related disease is lacking and in order to gain a better understanding, it will be necessary to develop diagnostic reagents and detection methods, particularly for sub-micronic particles. Monoclonal antibody-based assays enable the measurement of individual antigens but interpretation can be confounded by cross-reactivity between fungal species. The recent development of species-specific monoclonal antibodies, used in combination with a fluorescent-confocal HIA technique should, for the first time, enable the speciation of morphologically indiscernible fungal fragments. The application of this method will help to characterize the contribution of fungal fragments to adverse health effects due to fungi and provide patient-specific exposure and sensitization profiles. This will ultimately contribute to better patient management.


Airborne Fungal Fragments and Allergenicity

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Fungi are a diverse kingdom

- ~1.5 million species exist, ~80,000 species described.
- More than 112 fungal genera identified as allergenic.
- Relationship between fungal exposure and clinical outcomes remains unclear.
- Measurement of exposure to fungal allergens has been restricted to the spores of a select number of fungi.
- The potential of different fungi or fragments to cause or aggravate adverse health effects remains unclear.
- No consensus on thresholds for specific risks.
Fungi form mycelial networks of hyphae dispersed as spores or hyphal fragments.
Fungal fragments

• Derived from fragmented conidia or hyphae.

• Fungal fragments categorized into two groups depending on the analysis used.
  • 1. Submicron fungal fragments <1µm
  • 2. Larger fungal fragments >1µm

• Identification in environmental samples is complex and subjective.
Submicron fungal fragments

- Fragments of hyphae and conidia.
- Aerosolized from mycelium.
- Less than 1µm in size.
- Difficult to visualize and no visible morphological features.
- Most studies restricted to experimental environments.

Fragments and spores of *Aspergillus versicolor*

Fungal cultures release spores and smaller fragments

Immunological reactivity of fungal fragments

Aspergillus versicolor

Penicillium melinii

Larger fungal fragments

- Fragmented hyphae or conidia.
- Greater than 1µm in size.
- Fragmentation initiated by vacuolation.
- Easier to visualize compared to submicron fungal fragments but difficult to speciate.
- Contribute 6-56% of the total aerospora.

Environmentally sourced larger fungal fragment. Scale, 20µm.
Inhalation of larger fungal fragments

- Extent that larger fungal fragments were inhaled remained unknown.
- Intra-nasal air sampler developed by the Woolcock Institute of Medical Research.
- Interested in what fungi adults inhaled in an outdoor environmental setting.
- Results showed that personal exposure is highly variable between subjects and hyphal fragments contributed 9-13% of the total aerospora count.
- Personal exposure probably driven by physical disturbances.
Immunological reactivity of larger fungal fragments

• Despite many advances in understanding the contribution of fungi to respiratory diseases, the answers to many questions still remain elusive.

• Traditional methods for detecting airborne fungi are often unreliable and confounded by a number of variables.

• Recent technical advances have provided new insights into the nature of personal exposure to airborne fungi.

• The Halogen Immunoassay provides a method to match the spectrum of an individual’s allergic responses with the fungi that are collected in their own environment.
Germinated spore of *Epicoccum* spp. isolated from nasal washings and immunoprobed with fungal positive IgE using the Halogen Immunoassay.


Step 1: Collect airborne fungi onto protein binding membrane.

Step 2: Laminate membrane with adhesive coverslip and extract antigens.

Step 3: Detect antigens with primary monoclonal antibodies or human serum.

Step 4: Immunostain with secondary antibody conjugate.

Step 5: Visualize immunostaining using either (A) light microscopy or (B) confocal microscopy.

Immunoenzymatic human IgE staining (arrow a) of germinated (arrow b) Cladosporium herbarum conidia (arrow c).

Fluorescent mAb staining (arrow a) of Stachybotrys chartarum phialides.
Larger fungal fragments and allergenicity

- Conidia of a handful of species are primarily recognized as the distinctive fungal structures that function as sources of allergen.

- The extent that aerosolized hyphal fragments and other uncharacterized genera function as sources of allergen has remained unclear.

- Using the Halogen Immunoassay and a pool of fungal specific human serum, we explored the release of allergens from hyphal fragments collected from an indoor environment in Sydney, Australia.
Allergen release from larger fungal fragments

Not all larger fungal fragments release allergen

** P<0.0001

Hyphal fragments higher than conidia counts

** P<0.05
** P<0.0001

New fungal aeroallergen sources

Amphisphaeria spp.

Arthrinium spp.

Leptosphaeria spp.

Leptosphaerulina spp.

Myxomycete spores

Pleospora spp.

Spegazzinia spp.

Sporidesmium spp.

Ascomycete cleistothecium.

Xylariaceae ascospores

Limitations of the Halogen Immunoassay

• Unable to study allergen release from submicron fungal fragments, due to a number of limitations.

  – Difficult to differentiate between unicellular *Aspergillus* and *Penicillium* conidia.

  – Difficult to visualize and detect allergens from submicron fragments <1 µm in size.

  – Speciation of fungal fragments as sources of allergen is complex.
Dual Halogen Immunostaining

Extracted antigens
Germinated spore or fragment

Halogen assay…
germinate, laminate as previously

IgE
biotin-Goat αIgE
Alk-phos-Xavidin
NBT/BCIP (Alk Phos substrate)

α-fungal-species-M/P Ab
HRP-α-Mono/Poly Ab
Vector red (HRP substrate)

Alternaria alternata
Aspergillus fumigatus
Penicillium chrysogenum

Confocal Fluorescent Halogen Immunoassay

Extracted antigens

Spore or fragment

α-fungal-species mAb 6D4
Alexa Fluor® 488 goat anti-mouse IgM (green fluorescence)

Block membrane

α-fungal-species mAb 9B4
Alexa Fluor® 594 goat anti-mouse IgG (red fluorescence)

Immunostaining images captured using Confocal laser scanning microscopy

Conclusions

- Numbers of experimental or environmental fungal fragments are in most cases more common than airborne spores.

- Larger fungal fragments are a significant aeroallergen source, presenting a new paradigm of natural fungal exposure.

- Currently, the airborne distribution and immunoreactivity of smaller submicron fungal fragments remains unknown.

- The development of innovative immunodetection methods will help to elucidate adverse health effects due to smaller fungal fragments in a patient’s environment.
Future Perspectives

• Further develop the fluorescent Halogen Immunoassay format using confocal microscopy to enable the enumeration and speciation of environmentally sourced fungal fragments <1µm.

• Produce species-specific monoclonal antibodies for common indoor fungal species to speciate fungi in Halogen Immunoassays.

• Utilize the innovative immunodetection techniques in indoor and outdoor settings to characterize the airborne distribution and allergenicity of submicron fungal fragments.
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