# 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, ranging from subjective symptoms such as fatigue, cognitive difficulties or memory loss 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 various fungal components in environmental samples. Consequently, data on personal exposure and sensitization to fungal allergens are mainly based on the assessment of a few select and easily identifiable species. The contribution of other airborne spores, hyphae and fungal fragments 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. These fragments are particles derived from any intracellular or extracellular fungal structure and are categorized as either submicron particles or larger fungal fragments. *In vitro* studies have shown that submicron particles of several fungal species are aerosolized in much higher concentrations (300–500 times) than spores, and that respiratory deposition models suggest that such fragments of *Stachybotrys chartarum* may be deposited in 230–250 fold higher numbers than spores. The practical implications of these models are yet to be clarified for human exposure assessments and clinical disease. We have developed innovative immunodetection techniques to determine the extent to which larger fungal fragments, including hyphae and fractured conidia, function as aeroallergen sources. These techniques were based on the Halogen Immunoassay (HIA), an immunostaining technique that detects antigens associated with individual airborne particles >1 μm, with human serum immunoglobulin E (IgE). Our studies demonstrated that the numbers of total airborne hyphae were often significantly higher in concentration than conidia of individual allergenic genera. 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. Direct evidence of the associations between fungal fragments and

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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 submicron particles. Assays using monoclonal antibodies 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 novel 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.

## Introduction

The enormous diversity of the Fungal Kingdom is well recognized. It is estimated that more than a million species of fungi exist, and many of these produce airborne spores, conidia, hyphae or other fragments that can be inhaled by humans. About 112 fungal genera, belonging to three distinct taxonomic groups; the Ascomycota, Basidiomycota and the Deuteromycota are currently recognized to release allergens that exacerbate allergic diseases [1]. Fungal conidia and their associated components are unlike any other bioaerosol in that they are heterogeneous, biologically dynamic particles and are actively able to secrete molecules that have a variety of pathogenic, inflammatory and allergic properties.

Sensitization to fungi has been identified in multi-center studies to be a risk factor for severe asthma [2] and the prevalence of hypersensitivity to common fungal allergens among atopics has been estimated to range anywhere from as low as 2% to as high as 90% [2–12]. As with most allergens, reported variations in sensitization to fungi are dependent upon differences in exposures, the source and batch of commercial skin test extracts, the selection criteria of test subjects, and the methods of analysis. Understanding the relationship of exposure to clinical outcomes is further confounded by the subjective methods used to measure exposure such as spore counts, questionnaire surveys, and indoor assessments of visible fungal growth. For practical reasons, the measurement of personal exposure to fungal allergens has been restricted to a small select number of fungi including *Alternaria*, *Aspergillus*, *Penicillium* and *Cladosporium* species [13]. The reasons for this relate primarily to their airborne abundance in many geographic locations, their distinct morphological characteristics, and the availability of fungal extracts for diagnostic assays. This is particularly the case

for *Alternaria*, which has been widely studied in epidemiologic investigations [8,14–20]. Although fungal exposure in indoor environments has often been associated with health effects, the actual potential of different fungi to cause or aggravate such adverse health effects is still not clear. Furthermore, while numerous exposure guidelines for fungi have been proposed [21], there are no consensus threshold values [22]. The lack of such information is in part due to inadequacies of current monitoring techniques to establish accurate and precise measures of actual fungal exposure [23].

Research interests in aerosolized fungal fragments intensified following experimental and epidemiologic studies demonstrating the high concentrations of airborne fungal fragments [24,25] and their significance in relation to asthma severity [26]. Fungal fragments, as the term implies, are derived from broken or fractured conidia and hyphae that have been aerosolized following disturbances to fungal colonies. Differentiating between fungal and non-fungal fragments in environmental air samples is complex and subjective, however morphological features such as the presence of cross walls (septa) and melanization can be used. Currently, researchers categorize fungal fragments into either submicron particles ($<1$ μm) or larger fungal fragments ($>1$ μm) depending on the type of analysis.

We present here a brief review of the nature of fungal fragments, their allergenicity and new methods that have become available to detect these particles in environmental air samples.

## Fungal fragments in the environment

Numerous studies have investigated the extent that airborne fungal spores contaminate indoor environments, yet the prevalence and role of fungal fragments

to personal exposures has been rarely studied due to the difficulties enumerating and identifying these particulates. Several studies that have attempted to quantify larger hyphal fragments in indoor environments have shown that these particles represent a significant proportion of the aerospora, approximately 6–56% of the total fungal particle count [24,27–29]. It has also been shown that hyphal fragment counts do not correlate with total spore counts [27]. The aerosolization of these fragments is believed to originate from contaminated indoor surfaces [30,31] and a number of these particles may even retain their viability to facilitate the dispersal of the fungus [32]. When hyphal fragment counts have been incorporated into longitudinal epidemiological studies, associations with asthma severity and peak expiratory flow rates have been identified [26]. The concentration of fungi and the types of particles required to elicit respiratory health effects, however, remains unknown and controversial.

Although highly respirable particles ($<2.5$ μm) numerically constitute the majority of particulate matter found in indoor and outdoor air, the extent that smaller submicron fungal fragments contribute to the total particle load remains unclear due to the limitations of available methods of analysis [33,34]. These submicron particles have been the subject of current research to understand the processes involved in their aerosolization from contaminated surfaces and the concentrations of these particles in relation to total spore counts.

## Submicron fungal fragments

Submicron fungal fragments are any particles derived from intracellular and extracellular structures that have become aerosolized from fungal colonies [30,31]. These fragments are generally less than 1μm in size and lack distinct morphological features for their identification (Fig. 1A). As a consequence of the difficulties associated with collecting and identifying fungal fragments from contaminated environments, studies have been restricted to the analysis of a few fungal species under experimental conditions [30,31,35].

Using an aerosolization chamber developed by the group of Drs Tiina Reponen and Rafal Gorny for the standardized release and collection of bioaerosols [31,36,37], the authors studied the aerosolization of submicron fungal fragments from culture plates and ceiling tiles contaminated with *Aspergillus versicolor*, *Penicillium melinii, Cladosporium cladosporiodes,* and *Stachybotrys chartarum*. Fragments belonging to these species have been shown to aerosolize simultaneously with spores but at significantly higher concentrations (320–514 times higher) [31,35]. These findings have demonstrated that air velocity, time, colony structure, desiccation stress, moisture conditions and the degree of vibration may all influence the rate of aerosolization [30,31]. However, the process of aerosolization is believed to be quite different to that of spores and this is entirely dependent on the species and the type of surface (agar vs. ceiling tile). A thorough review of these mechanisms has been recently presented by Gorny [30].

In contrast, studies that have investigated the inhalation and respiratory deposition of submicron fungal fragments have been restricted to computer-based models. Cho and colleagues [35] recently demonstrated that the respiratory deposition of *S. chartarum* fragments was 230–250 fold higher than spores, although for *A. versicolor* the total counts were similar for spores and fragments. When the model was applied to infants it was demonstrated that the deposition ratios were 4–5 times higher than those for an adult [35]. The clinical consequences of these findings for personal fungal exposure are yet to be determined and require further investigation.

Information pertaining to the immunological reactivity of submicron fungal fragments is limited and

**Fig. 1** Photomicrographs of fungal fragments. Experimentally aerosolized *Aspergillus versicolor* submicron fungal fragments (A*) and a larger environmentally sourced fungal fragment (B, Scale bar, 20 μm). *Fig. 1A reprinted from Rafal Górny, Filamentous microorganisms and their fragments in indoor air – a review, *Ann Agric Environ Med* 2004; **11**: 185–197, with permission from the Institute of Agricultural Medicine, Lublin, Poland. 2006.
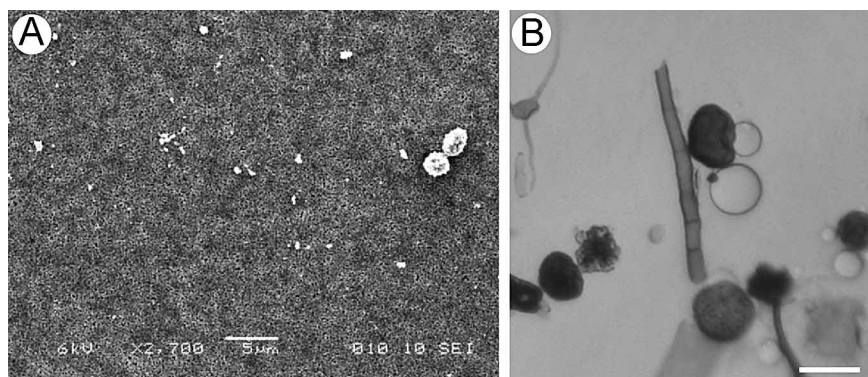
more difficult to obtain. This is mostly due to the difficulties involved with separating spores and fragments in air samples, in addition to the lack of available diagnostic reagents and detection techniques. Recent studies that have addressed these methodological limitations have shown that the immunological reactivity of fragments was anywhere from 2 to 5 times higher than spores, which was dependent on the species and type of monoclonal antibody used [31]. Likewise, Brasel *et al*. [38] demonstrated that macrocyclic tricothecene mycotoxins were detected not only in spores of *S. chartarum* but also in fragments, which were collected on polycarbonate filters (pore size 0.4μm). Moreover, the cytotoxic effects of submicron fungal fragments on *in vitro* cell lines have been recently explored. Reponen *et al*. [39] determined that exposure of mouse macrophage cell lines to fungal fragments caused the induction of pro-inflammatory mediators and the extent to which this occurred was dependent on the fungal species. Future studies using animal models are essential to determine the amount of submicron fragments required to elicit an adverse health response, the site of deposition within the lungs, and the innate and acquired immune mechanisms that are induced upon exposure.

To date, the allergenicity of submicron fungal fragments has not been studied in any detail due to the aforementioned methodological limitations. Although fungal fragments are abundant in the environment compared to spores and are small enough to be inhaled deep into the lungs, the degree that these fragments function as sources of allergens and contribute to adverse health effects has not been determined. Future studies should focus on the development of techniques that will enable the separation and purification of fungal fragments from other biological and non-biological particulates. Such studies will enhance the ability to detect and quantify these particles in experimental and environmental settings and test the health effects associated with exposure to separated and purified fungal fragments.

## Larger fungal fragments

Fungal fragments that are greater than 1 μm in size and comprise fragmented hyphae with visible septation or fractured conidia, have been classified as larger fungal fragments [28]. An example of a larger fungal fragment is depicted in Fig. 1B. Compared to submicron particles, larger fungal fragments are generally easier to visualize by light microscopy in environmental air samples and in some geographic locales their concen-

trations can account for up to 56% of the total aerospora load [24,27–29].

Compared to submicron fragments, the aerosolization of larger fungal fragments into the atmosphere has not been studied in as much detail. However, it is believed that this process is dependent on a number of similar variables, including wind speed, wind direction and substrate disturbance [28]. Several studies have shown that hyphal fragmentation is in fact a mode of reproduction for filamentous fungi [32]. Fragmentation is preceded by the formation of vacuoles following a reduction in available nutrients or environmental stress [40,41]. This process leads to the separation of hyphae at septal junctions and following wind dispersal allows their dissemination [42]. In contrast, the processes of conidial fragmentation have not been studied in detail and remain unknown. It is hypothesized that conidial fragmentation may be due to the rupturing of multicellular conidia along septal wall junctions or through osmotic pressure differences due to moisture differentials as previously described for grass and birch pollen [43–48], however this requires further investigation.

Recent environmental sampling collected by the Woolcock Institute of Medical Research (Sydney, Australia) using the intra-nasal air sampler (INAS) has provided some interesting insights into the extent that larger fungal fragments contribute to the inhalable fraction [49]. The INAS consists of a small plastic device, which can either be used as a personal air sampler [50–52] or a prophylactic personal filter device [53]. The device is worn in the nostrils and particles are impacted onto an internal adhesive surface, which is later removed for analysis. Monitoring results obtained with older versions of the INAS have been used successfully as proxy measures of personal exposure to inhaled dust mite [52], cat [54] and cockroach allergens [55]. More recently, a new prototype was developed that enables the collection of bioaerosols as small as 3 μm, which includes the spores and larger fragments of many airborne fungi [53]. In an analysis of the fungi inhaled by 34 adults in an outdoor setting [53], it was shown that the inhaled fungi were heterogeneous between subjects and sampling days [56]. In addition to larger hyphal fragments, spores of many species were identified including *Alternaria*, *Arthrinium*, *Bipolaris*, *Cladosporium*, *Curvularia*, *Epicoccum*, *Exserohilum*, *Fusarium*, *Pithomyces*, *Spegazzinia*, *Tetraploa* and Xylariaceae species. These results showed that inhalation exposure in most people is highly variable (2–10 fold differences) and that hyphal fragments contributed approximately 9–13% to the total fungal particle count. It was also shown that airborne fungal fragment counts collected by the INAS were

comparable to those collected by the personal Institute of Occupational Medicine sampler but higher than counts from a static Burkard spore trap located on top of a nearby building [56]. This study demonstrated that personal exposure to fungal spores and hyphal fragments is probably driven by physical disturbances, which aerosolize fungi from the soil surface into the breathing zone. Similar findings on the importance of physical disturbances for fungal aerosolization have been reported in studies from indoor environments. Butner and Stetzenbach [57,58] showed that human activity near the sampling site including walking on or vacuuming carpets, resulted in significantly higher concentrations of airborne spores compared to sampling under quiescent conditions. These findings were recently confirmed by Mitakakis *et al*. [51] using the INAS, who demonstrated that levels of inhaled *Alternaria* and *Cladosporium* spores were higher during periods of activity.

## Analysis of fungal fragments and allergenicity using the halogen immunoassay

Despite advances made in understanding the contribution of fungi to a range of respiratory diseases, the answers to many questions remain elusive. These include the temporal association between exposure to environmental fungi and respiratory outcomes, the identity of fungal components responsible for disease, and the molecules that participate in the interactions between fungal components and the patient.

Recent technical advances based on the Halogen Immunoassay (HIA) have provided new insights into the nature of personal exposure to airborne fungi [59,60]. This progress has not only resulted in personalized ambient exposure assessments but also raised entirely new aspects of fungal exposure such as the role of fungal germination on mucosal surfaces of the respiratory tract. However, the clinical impact of germination on the severity of the allergic response and the host immune response in general, remains to be characterized.

The HIA provides an innovative and powerful analytical tool to visualize proteins released from biological particles, such as fungal fragments. Unlike molecular techniques based on PCR, the HIA does not require the presence of any genetic material associated with the particles [28,60,61]. The principle of the assay is outlined in Fig. 2. Airborne particles are collected by suction (from cultures or air samples) onto a protein-binding membrane. Impacted fungal propagules are either allowed to germinate or processed immediately after collection. The sample is then permanently laminated using a thin adhesive-coated glass cover slip and the sandwich is immersed in buffer for several hours to allow the secretion and immobilization of proteins (antigens) in close proximity to the fungal particles on the membrane. Following blocking of vacant binding sites on the membrane, adsorbed antigens are detected with primary monoclonal antibodies for particle identification or human IgE for characterizing patient sensitization. The resulting
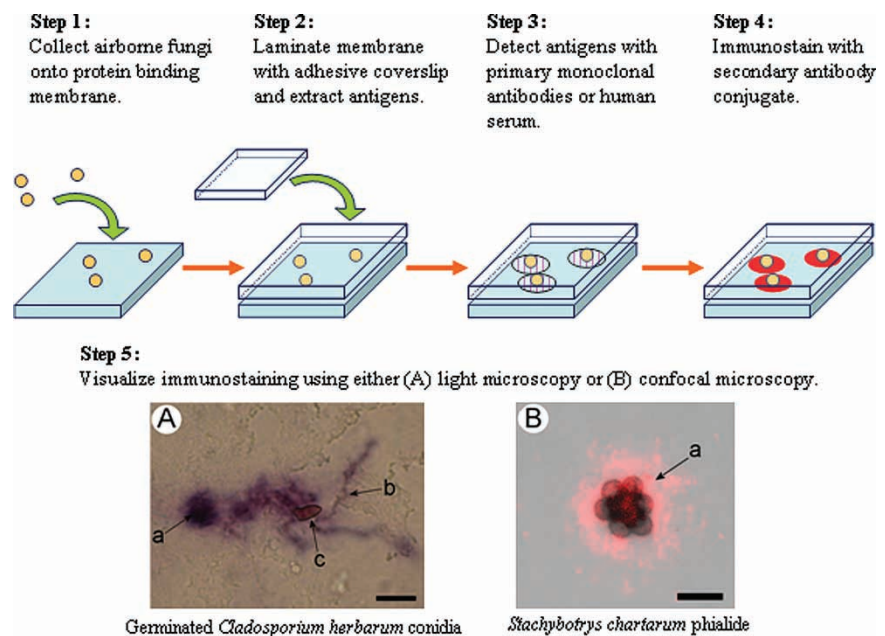


**Step 1:** Collect airborne fungi onto protein binding membrane.

**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.

Germinated *Cladosporium herbarum* conidia

*Stachybotrys chartarum* phialide

**Fig. 2** Experimental flow chart of the Halogen Immunoassay for fungi. (A) Immunoenzymatic human IgE staining (*arrow a*) of germinated (*arrow b*) *Cladosporium herbarum* conidia (*arrow c*). (B) Fluorescent monoclonal antibody immunostaining (*arrow a*) of a *Stachybotrys chartarum* phialide visualized using confocal laser scanning microscopy. Scale bar = 10 μm (A), 20 μm (B).

immune complexes are labeled with enzyme-conjugated or fluorophore-conjugated secondary antibodies and visualized using light, fluorescent, or confocal microscopy. Recent modifications of the HIA have additionally enabled the use of dual probes with different labels for different antibodies [62–64]. This allows the simultaneous visualization of antigens from multiple fungi in combination with serum IgE for a given patient (Fig. 3).

In addition to the analysis of environmental samples, the HIA can also be performed with a panel of culture-derived conidia to determine sensitization profiles of patients. As germination has been found to occur on respiratory mucosal surfaces after spore inhalation [65], the use of germinating spores rather than traditional fungal extracts may represent a more comprehensive source of allergens and in turn result in more accurate sensitization profiles. Recent studies, with single

allergens, have demonstrated that commercial fungal allergen extracts varied up to more than 400 fold for *A. fumigatus* extracts [66] and up to 3000 fold for *A. alternata* extracts [67]. Unlike conventional extraction-based methods to detect fungal allergy, HIAs provide a method to detect IgE to fungal particles directly obtained from the patient's environment [28,61]. The application of this methodology was recently demonstrated by testing the ability of the HIA to detect fungal allergic sensitization in a panel of atopic sera and comparing the results to those obtained with the UniCAP (CAP) system and *in vivo* skin prick test (SPT) [61]. This study showed that between 3–7% of negative sera were misdiagnosed by SPT but identified by both HIA and CAP to be sensitized to fungi. Furthermore, the HIA was concordant with CAP but there was significant discordance with SPT. The results of this study suggest
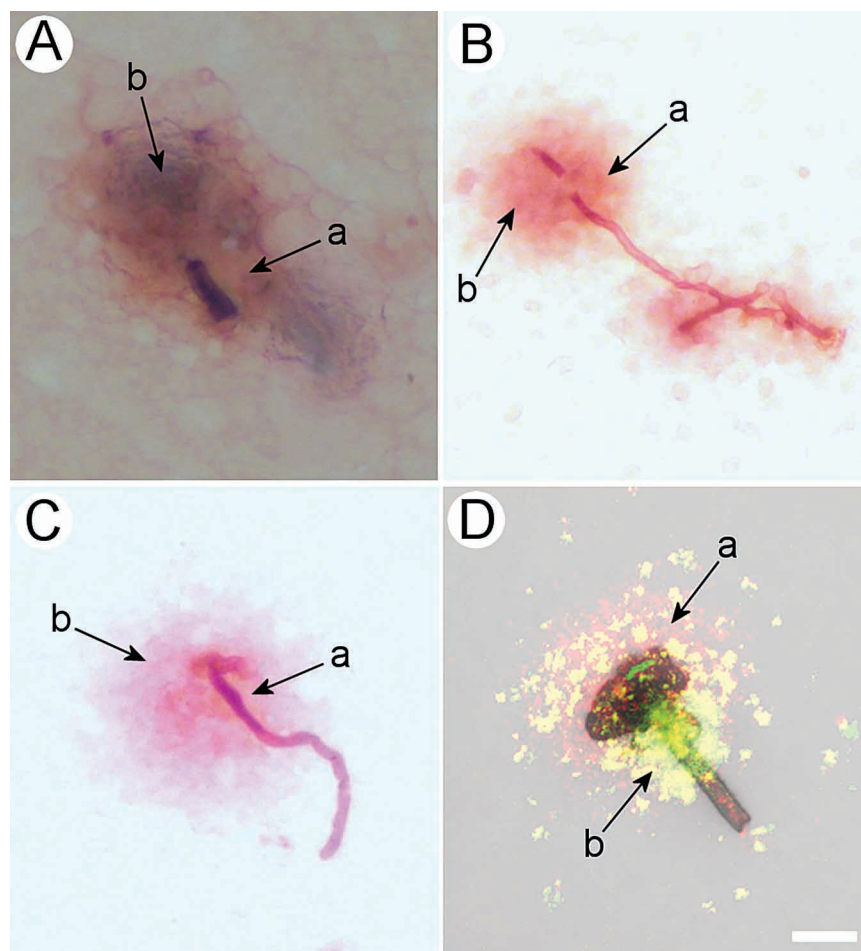


**Fig. 3** Dual enzymatic Halogen Immunostaining. (A*) Culture derived *Alternaria alternata* hyphal fragment (arrow a, polyclonal antibody raised against crude *Alternaria* extract, red precipitate; arrow b, pooled human serum IgE, purple precipitate), (B[†]) germinated *Aspergillus fumigatus* conidia (arrow a, monoclonal antibody 18G2, red precipitate; arrow b, pooled human serum IgE, purple precipitate), (C[†]) germinated *Penicillium chrysogenum* conidia (arrow a, monoclonal 18G2, red precipitate; arrow b, pooled human serum IgE, purple precipitate) and (D[‡]) high resolution image of dual fluorescent Halogen Immunoassay co-localized (yellow fluorescence) immunostaining of a *Stachybotrys chartarum* conidiophore with phialide using the monoclonal antibody 9B4 (arrow a, red fluorescence) and 6D4 (arrow b, green fluorescence). Scale bar = 10 μm. *Fig. 3A reprinted from Brett James Green, Detlef Schmechel and Euan Roger Tovey, Detection of aerosolized *Alternaria alternata* conidia, hyphae, and fragments by using a novel double-immunostaining technique, *Clin Diagn Lab Immunol* 2005; **12**: 1114–1116, with permission from the American Society for Microbiology, 2006. [†]Fig. 3B and C reprinted from Brett James Green, Detlef Schmechel, Jason Kingsley Sercombe and Euan Roger Tovey, Enumeration and detection of aerosolized *Aspergillus fumigatus* and *Penicillium chrysogenum* conidia and hyphae using a novel double immunostaining technique, *J Immunol Meth* 2005; **307**: 124–137, with permission from Elsevier, 2006. [‡]Fig. 3D reprinted from Brett J. Green, Lyndell L. Millecchia, Francoise M. Blachere, Euan R. Tovey, Donald H. Beezhold and Detlef Schmechel, Dual fluorescent halogen immunoassay for bioaerosols using confocal microscopy, *Anal Biochem* 2006: 354: 151–153, with permission from Elsevier.

**Fig. 4** Halogen immunostaining of airborne fungi and associated environmental fragments using a human serum IgE pool from fungal-sensitive subjects. Resultant immunostaining of (A*) airborne hyphal fragment with immunostaining restricted around the hyphal tips (arrow a) and (B*) immunostaining restricted to the site of conidial fragmentation (arrow a). *Fig. 4A and B reprinted from Brett James Green, Jason Kingsley Sercombe and Euan Roger Tovey, Fungal fragments and undocumented conidia function as new aeroallergen sources, *J Allergy Clin Immunol* 2005; **115**: 1043–1048, with permission from Elsevier.
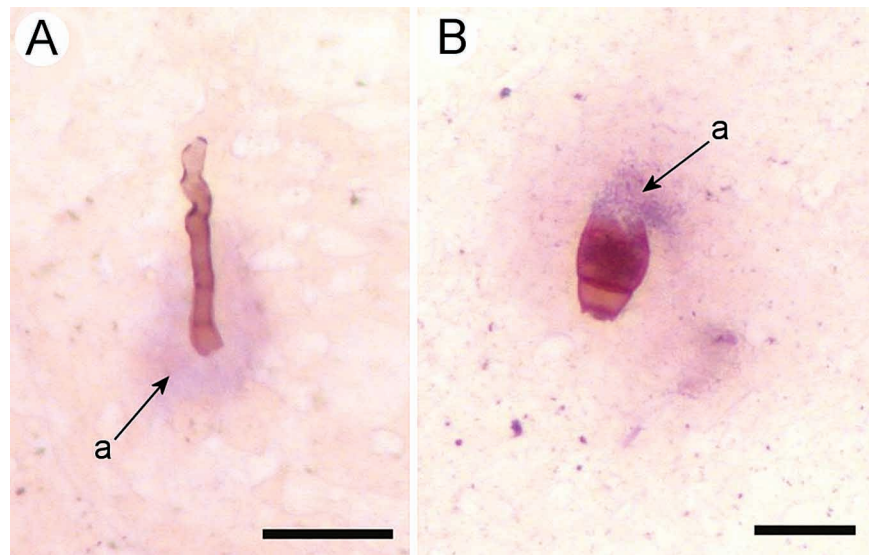
that the spectrum of allergens actively released by the spores, hyphae, and fragments in the HIA may be more analogous to those actually occurring during natural human exposure. Thus, the HIA potentially provides a more accurate measure of fungal sensitization.

Using the HIA and a pool of fungal-specific human serum, it was possible for the first time to explore the release of allergens from hyphal fragments collected from an indoor environment [28]. Although the results of this kind of study would be expected to vary according to the geographic location and specificity of the human IgE, it was clear that not all hyphae released allergens. In our study, approximately 25% of hyphal fragments expressed detectable allergen in the HIA [28]. The reasons for this variation remain unclear but may be due to allergen denaturation following exposure to ultraviolet light, high temperatures or excessive moisture.

In the same study [28], it was shown that the localization and intensity of immunostaining was heterogeneous among the larger hyphal fragments [28]. The majority of fragments demonstrated significant IgE binding to allergens around the hyphal tips. There were several examples of staining confined to the septal junctions or around the entire fragment. An example of a larger fungal fragment with an associated halo of immunostaining is shown in Fig. 4A. It appears however, that intracellular allergens are preferentially released from the terminal ends of mycelial fragments and that fragmentation coincides with regions of septation [28]. The intense staining of hyphal fragments suggests that these particles need to be considered as

important additional sources of fungal allergens that should be included in environmental analyses.

Fragmented conidia were also found to release allergens, although their numbers were comparatively lower than immunostained hyphal fragments [28]. The localization of IgE binding to expressed allergens was primarily at the site of fragmentation, often in higher concentrations than intact conidia belonging to the same species (Fig. 4B). Furthermore, these studies also showed that the diversity of fungal species recognized by human IgE was far greater than previously reported. Using the HIA, ten previously uncharacterized fungal genera were identified and accounted for 8% of the total conidia that demonstrate IgE binding. These findings not only demonstrate the limitations of current monitoring methods but also emphasize the need to incorporate fungal fragments and a much wider range of fungal species into environmental analyses, thus representing a new paradigm of fungal exposure assessment.

At present, it has not been possible to use the HIA to study allergen release from submicron fungal fragments or quantify their aeroallergen load, due to the detection limits associated with current enzyme-based immunostaining techniques. However, the need to quantify submicron fragments has recently been pointed out by Cho and colleagues [35], who demonstrated using respiratory deposition models, that smaller fungal fragments can penetrate much deeper into the lung than larger conidia. Although the biological importance of submicron fungal particles remains unknown, previous studies assessing pollen exposure have established pollen fragments to be major causes in 'thunderstorm asthma' [43,45,68]. Similar research is required
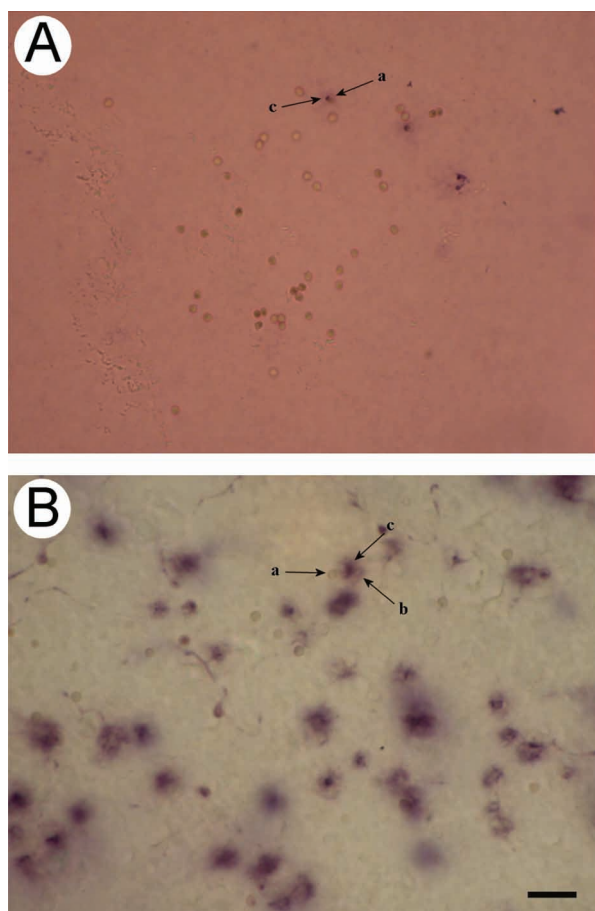
**Fig. 5** (A) Individual ungerminated (arrow a) and (B) germinated *Aspergillus fumigatus* conidia (arrow b) immunostained by the Halogen Immunoassay with human serum IgE (arrow c). Scale bar =10 μm.

**Fig. 6** *In vitro* effects of simulated nasal cavity temperature profiles on the rate of conidial germination (A and C) and hyphal growth (B and D) of *Epicoccum nigrum* (A and B) and *Bipolaris spicifera* (C and D). The temperature values correspond to the vestibule (25.3°C, ●), middle turbinate (32.3°C, ○), nasopharynx (33.9°C, ▼) and the mainstem bronchi (36.9, △) as determined by Keck *et al.* [81] and McFadden *et al.* [82]. Conidia of *E. nigrum* and *B. spicifera* were incubated in dH$_2$O in a concavity well microscope slide under moist conditions. The proportion of conidia with an observable germ tube and germ tube length was measured at 0, 1, 3, 5, 7, 10 and 20 hour intervals. Values presented for A and C represent the mean (± SE) proportion determined from counting the number of germinated conidia from a total count of all conidia present. A minimum of 100 conidia were counted per sample. The values presented for B and D represent the mean (± SE) hyphal growth rate determined from measuring the length of hyphae at each time interval. Six replicates were evaluated, and the experiment was repeated.
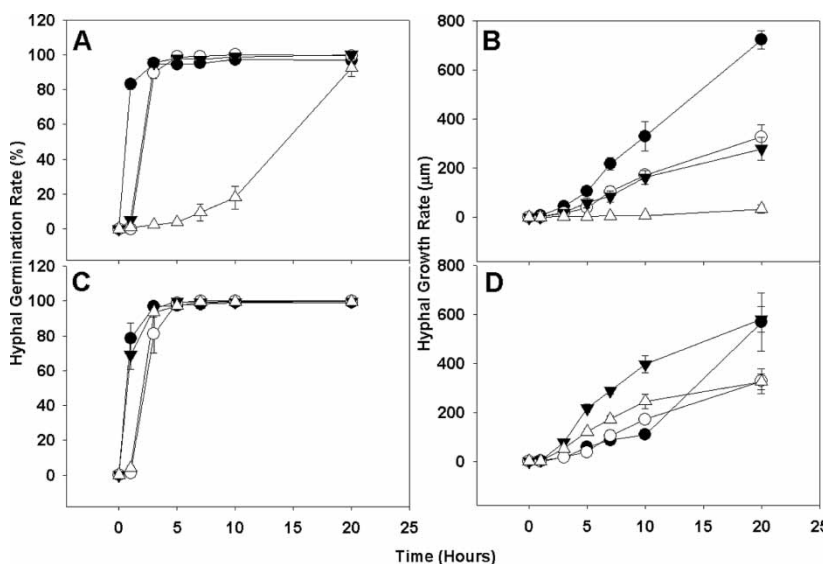


to determine the clinical relevance of exposure to fungal fragments.

## *In vivo* fungal germination and increased allergen release

Unlike other carriers of common allergens, such as pollen grains or mite feces, fungal spores and hyphae initially release only small quantities of allergen following inhalation and deposition in the respiratory tract. This initial allergen release also does not require the conidia to be viable [69]. However, preliminary nasal lavage studies have demonstrated that fungal conidia belonging to *Aspergillus, Penicillium,* and *Alternaria* species that were inhaled under conditions of natural exposure were in a state of germination [65]. The quantity of allergen released during germination increases with time [70–74] and the dynamics of this process have been shown, *in vitro,* to differ between species [73,74]. An example of allergen detection before and after germination of *A. fumigatus* conidia is shown in Fig. 5.

In addition, the rate of germination may also depend on the immune status of the patient, the anatomical site of deposition within the lung as well as the micro-environmental conditions within the respiratory tract, such as temperature and moisture gradients. For instance, preliminary *in vitro* germination and growth experiments have shown that *Epicoccum nigrum* has slower rates of germination and hyphal growth at higher temperatures (>30°C) compared to *Bipolaris spicifera* (Fig. 6). Thus, it can be expected that certain

fungal species will have the ability to initiate germination in certain regions of the respiratory tract. This may be why researchers only isolate thermotolerant *Aspergillus* and *Bipolaris* species in cases of chronic sinusitis and invasive Aspergillosis [75–80]. Thus, the consequences of fungal exposure may not only be a function of the number and diversity of fungal particles but also depend on the degree of fungal germination and the efficiency of the local innate immune system to overcome virulence factors released by germinating fungi.

## Conclusions and future perspectives

Studies that have focused on sources of experimental or environmental fungal fragments indicate that these particles are more common than airborne spores and may function as sources of allergen. These findings have presented a new paradigm of natural fungal exposure implicating the additional contribution of fungal hyphae, fragmented conidia, and previously overlooked fungal genera to personal exposure and fungal allergy. However, even with the recent advances of immunodetection techniques, the extent that smaller submicron fragments function as sources of allergen remains to be determined. This is of particular interest as smaller fragments may be deposited much deeper within the respiratory tract than intact fungal conidia and larger hyphal fragments. Such studies will help to elucidate adverse health effects due to fungal aerosols and ultimately contribute to better patient management.

Future research will concentrate on the further development of fluorescent-based HIAs using confocal laser scanning microscopy to detect submicron fungal fragments in environmental air samples [62]. This technique will improve particle resolution and sample contrast as well as provide for the possibility of quantitative image analysis and automated sample processing. Furthermore, current projects aim at the production of species-specific monoclonal antibodies to a wider range of fungi commonly found in occupational and indoor environments. The availability of these antibodies in combination with HIA-based immunoassays will allow a more comprehensive assessment of fungal exposure in the future.

## Acknowledgements

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