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Research paper

Enumeration and detection of aerosolized *Aspergillus fumigatus* and *Penicillium chrysogenum* conidia and hyphae using a novel double immunostaining technique

Brett James Green [a,c,*], Detlef Schmechel [c], Jason Kingsley Sercombe [b], Euan Roger Tovey [a,b]

[a] *Department of Medicine, The University of Sydney, NSW, Australia*
[b] *Woolcock Institute of Medical Research, Sydney, NSW, Australia*
[c] *National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, 1095 Willowdale Road, M.S 4020, Morgantown, WV 26505-2888, United States*

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Abstract

The identification of collected airborne unicellular fungal conidia and hyphae using nonviable techniques is subjective and an imprecise process. Similarly, to determine whether an individual is allergic to a particular genus requires a separate immunodiagnostic analysis. This study demonstrates the development of a novel double immunostaining halogen assay, which enables (1) the simultaneous identification of collected airborne fungal conidia and hyphae of *Aspergillus fumigatus* and *Penicillium chrysogenum* using monoclonal antibodies and (2) the demonstration of patient-specific allergy to the same particles using human serum IgE. The results demonstrate that when conidia were ungerminated the binding of antibodies was homogeneous and localized in close proximity around the entire conidia for both species. However, when conidia were germinated, the proportion expressing antigen increased ($P < 0.0001$) for both species and the sites of binding of the two antibodies changed with double immunostaining restricted to the hyphal tips for *A. fumigatus*, in addition to the sites of germination for *P. chrysogenum*. The described immunoassay has the potential to identify fungal particles in personal environmental air samples, provided species-specific monoclonal antibodies are available, while simultaneously demonstrating allergic sensitization to the same particles by co-staining the samples with the patient's own serum. Such an immunoassay can use those fungi that the patient is actually exposed to and potentially avoids many problems associated with extract variability based on the performance of current diagnostic techniques for fungal allergy. Published by Elsevier B.V.

Keywords: Allergen; Conidia; Fungi; Germination; Immunoassay; Mold

1. Introduction

Personal exposure to airborne fungi is recognized to be a risk factor for seasonal rhinitis (Li and Kendrick, 1995), asthma (Downs et al., 2001) and even death (O'Hollaren et al., 1991). The collection and enumeration of airborne fungal conidia, hyphae and more recently fungal fragments in bioaerosol and occupational

Abbreviations: HIA, halogen immunoassay; IgE, immunoglobulin E; mAb, monoclonal antibody; SPT, skin prick test; MCE, mixed cellulose ester; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HRP, horseradish peroxidase.

* Corresponding author. National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, 1095 Willowdale Road, M.S 4020, Morgantown, WV 26505-2888, United States. Tel.: +1 304 285 5884; fax: +1 304 285 6126.
E-mail address: Brett.Green@cdc.hhs.gov (B.J. Green).

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investigations is complex. Various viable and nonviable sampling techniques are available but are often confounded by a lack of specificity, long incubation times and subjective identification methods. These limitations are in part due to the viability of conidia, which prevent the detection in culture-based techniques, the lack of suitable DNA probes for molecular techniques and the inability to speciate small conidia and morphologically indiscernible fragments using light microscopy (Rogers, 2003; Schmechel et al., 2003a). Furthermore, current in vitro methods to diagnose allergy to fungi are restricted by the availability and variability of allergen extracts (Esch, 2004). It is likely that fungal allergy is both under-diagnosed and airborne fungi are incorrectly identified as causes of allergic symptoms.

Not since the integration of direct microscopy and immunohistochemistry (Popp et al., 1988) has it been possible to collect and enumerate airborne wild-type fungal particles and concurrently demonstrate antigen–antibody interactions. Such immunohistochemical techniques, however, are confined to only recognizing surface antigens fixed in or on the particles themselves. The development of the halogen immunoassay (HIA) has permitted the co-visualization of individual fungal conidia and hyphae collected by volumetric air sampling together with their expressed antigens immunostained around the particle as a halo with human immunoglobulin E (IgE) (Tovey et al., 2000). However, the identification of the small (2–3 μm) and unicellular *Aspergillus* and *Penicillium* conidia and hyphae collected onto protein binding membranes has previously been based entirely on conidial morphological criteria and to date remains subjective (Green et al., 2003). In this, proof of principle, study we describe a novel double immunostaining technique using the HIA, that permits the enumeration and identification of culturally derived unicellular *Aspergillus fumigatus* and *Penicillium chrysogenum* conidia and hyphae with monoclonal antibodies (mAbs) and the concurrent immunostaining of allergens with human IgE to the same fungal propagules.

2. Materials and methods

2.1. Culture, aerosolization and collection of fungal conidia

Fungal isolates of *A. fumigatus* (28 004) and *P. chrysogenum* (28 002) were supplied by the Queensland Department of Primary Industries (Brisbane, Australia). The isolates were sub-cultured from stock sources and grown for 10 days on vegetable juice nutrient agar at 24.9 °C. Conidia were aerosolized from sporulating cultures by use of an air jet and then collected by suction onto a mixed cellulose ester (MCE) protein binding membrane (0.8 μm pore size; Millipore Corporation, Bridgewater, MA) as described previously (Green et al., 2003). To germinate conidia, the membrane was moistened in deionized water and placed in a humid box for 12 h at 24.9 °C to allow germination. Both germinated and ungerminated conidia samples were permanently laminated to the MCE by overlaying it with a glass coverslip that had been pre-coated with a film of optically clear adhesive (Woolcock Institute of Medical Research, Sydney, Australia).

2.2. Human serum samples

Human sera from 30 subjects with asthma who were allergic to *Alternaria* and other fungal genera were collected and pooled. The diagnosis was based upon a documented clinical history of asthma, and allergy was determined by a positive SPT with a wheal diameter of 3 mm or greater. Specific IgE towards a panel of fungal allergens was detected in the pool by Pharmacia UniCAP (Pharmacia, Uppsala, Sweden) (Pharmacia CAP score; specific IgE to *A. alternata* = 60.7 kU_A/l). All samples were stored in aliquots for future use at −70 °C. Pooled serum IgE from 10 subjects, SPT negative to fungi but sensitized to other non-fungal allergens (Pharmacia CAP score; specific IgE to *A. alternata* <0.35 kU_A/l), in addition to an in-house rabbit polyclonal antibody raised against a crude *Lolium perenne* pollen extract were used as a negative control (Green et al., 2003). The local research ethics committee approved the study protocol and the subjects gave written informed consent following a full explanation of the study.

2.3. Monoclonal antibody production

Briefly, mice were immunized with extracts of *P. chrysogenum* conidia as described previously for *Aspergillus versicolor* (Schmechel et al., 2003b). The mAb 18G2 (IgG1) was found to extensively cross-react with the mycelium or spores of a number of fungal species commonly identified in indoor environments, including *A. niger*, *A. versicolor*, *P. citrinum*, *P. roqueforti*, *P. brevicompactum* and mycelium of *A. fumigatus*.

2.4. Immunostaining

Previously laminated ungerminated or germinated conidia (from Section 2.1 above) were immersed in

borate buffer (pH 8.2) for 4 h to enable antigens and other macromolecules to elute and bind in close proximity to the conidia and hyphae on the membrane. Unoccupied sites on membranes were blocked in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 45 min and then incubated overnight with a positive IgE serum pool diluted 1/3 in 1% BSA–PBS–0.05% Tween 20. After the primary antibody incubation, the membranes were rinsed three times in PBS–0.05% Tween 20 and incubated for 1.5 h with biotinylated goat anti-human IgE (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted 1/500 in 1% BSA–PBS–0.05% Tween 20. This was followed by an incubation for 1.5 h with a mouse mAb 18G2 (tissue culture supernatant) raised against *P. chrysogenum* (National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Morgantown, WV), which was diluted 1/5 in 1% BSA–PBS–0.05% Tween 20. Following the mAb incubation, the membranes were rinsed and then incubated for 1.5 h with an anti-mouse IgG horseradish peroxidase (HRP) conjugate (Sigma Chemical Co, St Louis, MO). Finally, biotinylated anti-human IgE antibodies were labeled with an ExtrAvidin alkaline phosphatase conjugate (Sigma Chemical Co, St Louis, MO) diluted 1/1000 in 1% BSA–PBS–0.05% Tween 20 and incubated for 1.5

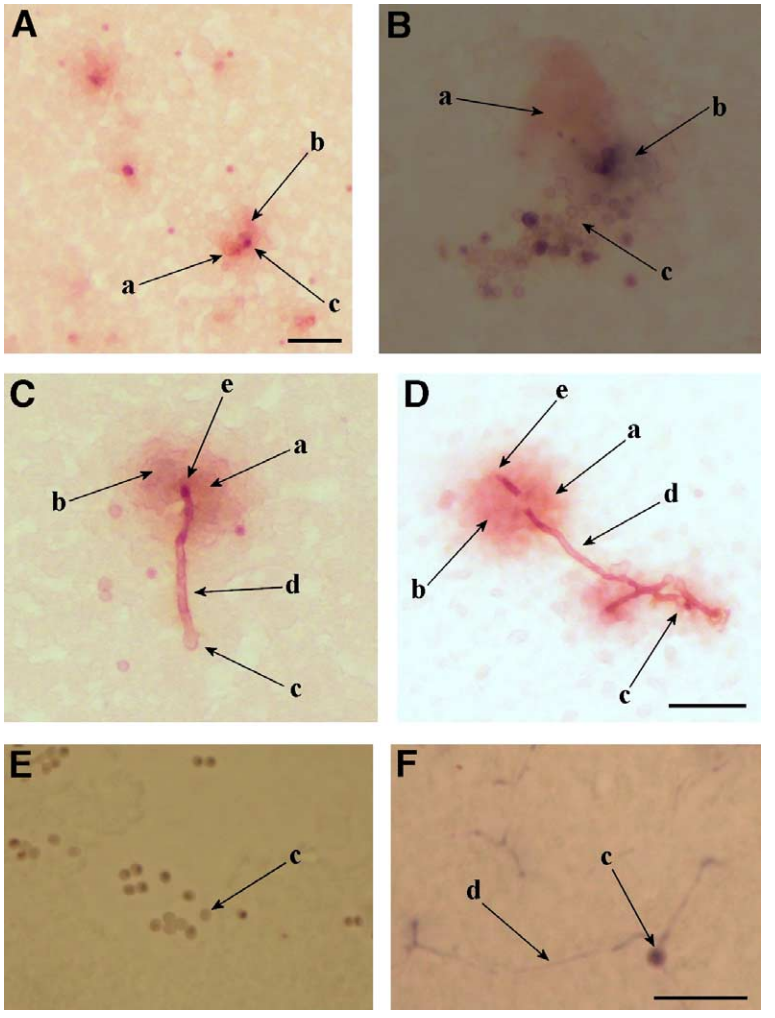

Fig. 1. Dual immunostaining of culture-derived *A. fumigatus* conidia (A–B) and germinated conidia (C–D) using mAb 18G2 (arrow a; red precipitate) and human serum IgE (arrow b; purple precipitate). Immunostaining was confined to (A) around the entire conidia (arrow c), (B) regions of clustered conidia (arrow c) and (C–D) around the hyphae and hyphal tips (arrows d and e) of germinated conidia (arrow c). (E–F) Negative controls using a pool of sera from atopic but *Alternaria* negative subjects (Pharmacia CAP score; specific IgE to *A. alternata* <0.35 kU_A/l) and an in-house rabbit pAb raised against a crude *L. perenne* pollen extract showed no localized immunostaining around the conidia (arrow c) and hyphae (arrow d). Scale bar, 20 μm.

h. The membranes were then rinsed three times in PBS–0.05% Tween 20 before being developed. Assay conditions such as antibody concentrations and incubation times were selected to optimize the staining of the mAb in combination with the serum human IgE.

For immunostaining, Vector NovaRED substrate for HRP (Vector Laboratories, Burlingame, CA) was first prepared as per the manufacturer's instructions and samples were incubated with the substrate for approximately 1 h to allow adequate development of the red precipitate. The membrane was then rinsed three times in PBS–0.05% Tween 20 and transferred to a separate staining well containing the alkaline phosphatase NBT/BCIP substrate (Pierce Chemical Co, Rockford, IL). Staining was then monitored periodically for approximately 20 min until an optimum dark blue precipitate was achieved. Entire membranes were then examined at a magnification of 200× by using standard light microscopy as described previously (Green et al., 2003) and double immunostained conidia and hyphae were counted by an unblinded examiner. Three replicates were evaluated and the experiment was repeated with

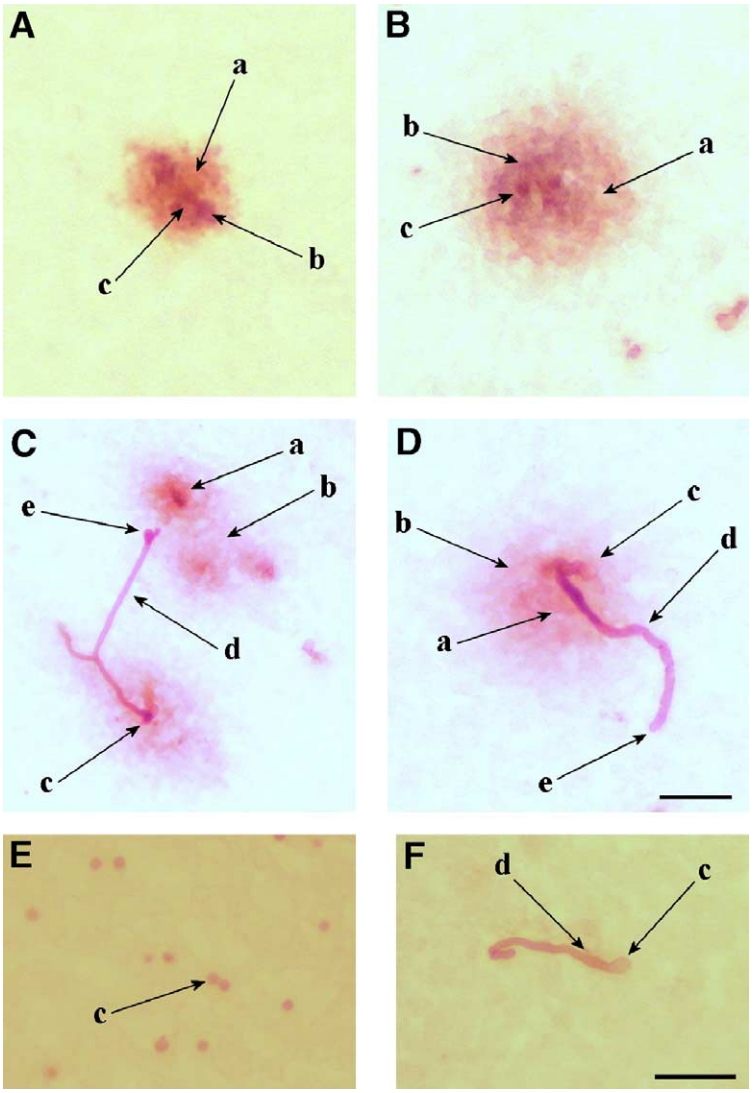

Fig. 2. Dual immunostaining of culture-derived *P. chrysogenum* conidia (A–B) and germinated conidia (C–D) using mAb 18G2 (arrow a; red precipitate) and human serum IgE (arrow b; purple precipitate). Immunostaining was confined to (A–B) around the entire conidia and (C) around the periphery of the hyphal tips (arrows d and e), as well as (D) in close proximity to the site of conidial germination (arrow c). (E–F) Negative controls using a pool of sera from atopic but *Alternaria* negative subjects (Pharmacia CAP score; specific IgE to *A. alternata* <0.35 kUA/l) and an in-house rabbit pAb raised against a crude *L. perenne* pollen extract showed no localized immunostaining around the conidia (arrow c) and hyphae (arrow d). Scale bar, 20 μm.

appropriate controls, including the rabbit polyclonal antibody raised against a crude *L. perenne* pollen extract and the pooled adult human sera SPT negative to fungi but sensitized to other non-fungal allergens.

2.5. Statistical analysis

Differences between mean values for double immunostained subsets among ungerminated and germinated groups were analysed for significance using the Independent samples *t*-test (Analyse-It for Microsoft Excel, Version 1.68, Analyse-It Software Ltd., Leeds, United Kingdom). The criterion for significance for all analyses was $P<0.0001$. Except where otherwise noted, all data are expressed as mean proportions ± range.

3. Results

The observed antigen immunostaining as detected by the mAb and the human IgE differed between each of the species and the state of germination (Figs. 1 and 2). For ungerminated conidia belonging to *A. fumigatus*, the resultant mAb immunostaining was primarily localized as a red halo around the outer extremities and on either margin of the conidia, whereas the resultant IgE immunostaining was in close proximity around the entire conidia and appeared as a dark purple halo (Fig. 1A). In addition, significant concentrations of antigen were expressed in regions of clustered *A. fumigatus* conidia (Fig. 1B). For *P. chrysogenum*, the mAb and IgE immunostaining were localized around the perimeter of the conidia and staining by both antibodies was homogeneously distributed (Fig. 2A and B), often in greater concentrations compared to *A. fumigatus*.

Upon germination, greater concentrations of both mAb and IgE immunostaining were observed with both species, when compared to the ungerminated conidia. Similar patterns of antigen immunostaining were evident for *A. fumigatus* (Fig. 1C and D) and *P. chrysogenum* (Fig. 2C and D). However, a number of subtle variations existed between the genera. For *A. fumigatus*, the resultant mAb and IgE immunostaining was localized in the same regions around the growing hyphal tips of the germinated conidia (Fig. 1C and D). However, for *P. chrysogenum* the resultant mAb and IgE immunostaining was heterogeneous and primarily localized in the vicinity of growing hyphal tips (Fig. 2C and D), in addition to around the area of conidial germination (Fig. 2C and D). For *A. fumigatus*, there was greater staining of the hyphal structure itself by the mAb (Fig. 1C and D), whereas with *P. chrysogenum*, the hyphae showed greater direct staining with IgE (Fig. 2C and D). In addition, no antigen immunostaining was detected in both mAb and human IgE negative controls (Figs. 1E,F and 2E,F).

Table 1
Summary of the proportion of ungerminated and germinated *A. fumigatus* and *P. chrysogenum* conidia treatments demonstrating double immunostaining with mAb and human IgE

State of fungal germination	Mean proportion of fungal conidia (range)[a]	
	No immunostaining	mAb+IgE immunostaining
Aspergillus fumigatus[b]		
Ungerminated conidia	68.8 (60.4–73.3)	31.2 (26.7–39.5)
Germinated conidia	58.3 (44.5–67.1)	**41.7 (32.9–55.5)**[c]
Penicillium chrysogenum[d]		
Ungerminated conidia	86.1 (82.9–13.9)	13.9 (18.6–17.1)
Germinated conidia	19.5 (11.8–31.4)	**80.4 (68.6–88.2)**[c]

[a] Values presented represent the mean determined from counting the number of ungerminated and germinated conidia expressing double immunostaining from a total count of all conidia present. In some instances, under development of the precipitating stains may have under-represented the proportion of conidia demonstrating double immunostaining.
[b] All *A. fumigatus* negative control treatments showed no immunostaining around the ungerminated conidia and germinated conidia and hyphae.
[c] Bold type identifies the germinated conidia double immunostained values, which are significantly higher than those double immunostained in the ungerminated conidia treatment ($P<0.0001$).
[d] All *P. chrysogenum* negative control treatments showed no immunostaining around the ungerminated conidia and germinated conidia and hyphae.

The proportion of ungerminated and germinated conidia demonstrating double immunostaining for each of the species is presented in Table 1. Approximately 31% of *A. fumigatus* and 14% of *P. chrysogenum* ungerminated conidia expressed dual mAb and human IgE immunostaining (Table 1). Upon conidial germination, however, the percentage showing double immunostaining (Table 1) rose to 42% and 80%, respectively ($P<0.0001$).

4. Discussion

Traditional methods of sampling airborne fungi have been confounded by a number of limitations, which has made data interpretation and comparisons between studies difficult. Viable culture detects only those fungi that are capable of growth on the selected nutrient medium, while other factors including environmental conditions, sampling practices, transport of materials and culture conditions also influence the final outcome (Stetzenbach

et al., 2004). Alternatively, nonviable methodologies are often subjective and imprecise with recent estimates of up to 50% of airborne fungi in aerobiological investigations misclassified (Flannigan, 1997). The recent development of more reliable nonviable methods have corresponded with the advances in molecular biological techniques, which have seen the development of PCR (Haugland et al., 2004; Vesper et al., 2004) and DNA hybridization probes (Wu et al., 2002) as well as recombinant mAbs specific to individual fungal antigens, the most common including Alt a 1 and Asp f 1 (Vailes et al., 2001). These mAbs have permitted the quantification of specific fungal allergens in environmental air samples using immunoassays, as well as demonstrated the localization of allergens on the surface of conidia and hyphae in immunohistochemical studies (Reijula et al., 1992). However, even with these molecular advances the enumeration and identification of collected wild-type unicellular fungal conidia and hyphae continues to be taxonomically challenging. Currently there are no techniques which allow the identification and simultaneous demonstration of allergic sensitization to the same collected airborne wild-type fungal propagules.

While immunoassay techniques for the quantification of antigens and the identification of cells have found wide applications within many areas of biology, their contribution to mycology has, so far, been less productive. The available immunohistochemical techniques based on the staining of surface structures (Popp et al., 1988; Reijula et al., 1992) are not practical for general use, while immunoassays of some soluble allergens from conidia are confounded by the production of such allergens by different fungal genera (Bisht et al., 2002). Halogen immunoassays, particularly when used with this double immunostaining technique, provide novel insights. They allow the application of traditional morphological techniques to identify structures and combine this with the ability of monoclonal or polyclonal antibodies to further identify conidia along with the use of a second human antibody to diagnose whether an individual is allergic to that particular species.

Our proof of principle study has demonstrated that the localization of the antigens detected by both primary antibodies was heterogeneous between the two fungal genera and varied between the states of germination. Although conidial structure and allergen function were not investigated in the present study, the heterogeneous patterns of antigen expression for *A. fumigatus* and *P. chrysogenum* suggest that morphological characteristics of the conidial walls are important features in determining the resultant double immunostaining of expressed antigens in the HIA. The expression of fungal antigens from intact conidia and hyphae has been poorly investigated. However, several studies using thin-sectioning and immunoelectron microscopy have demonstrated the location of allergens belonging to *Cladosporium cladosporioides* (Bouziane et al., 1989) and *A. fumigatus* (Reijula et al., 1991). Based on these reports, the cell wall, cell membrane (Bouziane et al., 1989) and to a lesser extent, the cytoplasm (Reijula et al., 1991) are the principal locations in which allergens are expressed in the conidia. In addition, the conidia of a number of airborne fungi are covered by an interwoven fibrous network of rodlets, which have been identified by immunoassays to contain the highest concentrations of conidial allergen and function to regulate the permeability of many cytoplasmic components, including antigen-presenting macromolecules (Bouziane et al., 1989).

Several experimental studies have also demonstrated that the germination of *Aspergillus* (Sporik et al., 1993), *Alternaria* (Mitakakis et al., 2001) and a number of other fungal genera (Green et al., 2003) increase the amount of detectable allergen. Newly emerged hyphal tips from germinated conidia are functionally differentiating and metabolically active regions of the hyphae which have been shown to bind considerably greater amounts of antibody compared to conidia (Reijula et al., 1991). In the current study, fewer ungerminated *A. fumigatus* and *P. chrysogenum* conidia were detected than germinated conidia. Furthermore, the germination of conidia increased the amount of expressed antigen and when compared to ungerminated conidia, provided a greater concentration of double immunostaining. Unicellular *Aspergillus* and *Penicillium* conidia share small dimensions (<3 μm) and, compared to hyphae, contain only negligible quantities of antigen-bearing macromolecules. These amounts of antigen (probably in the low pg quantity per conidial) impose a lower limit of detection on the assay. Future studies should explore ways to improve the detection thresholds. This would further clarify wild-type fungal conidia as well as permit the resultant immunostaining to be semi-quantitatively analysed. Recent techniques of quantitative immunohistochemistry will be explored to address the possibility of quantifying fluorescent signals and counting fluorescently labeled fungal particles in experimental and environmental samples (Kirkeby and Thomsen, 2005). However, to date, this has not been possible using current materials as preliminary tests reveal that a combination of available fluorophores and precipitating substrates were less sensitive and confounded by quenching (unpublished data).

In addition to identifying fungal conidia, the HIA has been applied to the identification of several airborne allergens, including hyphae and morphologically indiscernible fungal fragments in environmental air samples (Green et al., 2005). These studies have shown the feasibility of this technique to successfully characterize fungal exposures.

Aerosolization studies have demonstrated that fungal fragments and hyphae are significantly higher in concentration than airborne conidia of any single species (Gorny et al., 2002; Green et al., 2005) and that these fragments released detectable quantities of allergen (Green et al., 2005). However, the identification and quantification of such particles using nonviable methods has not been possible to date, using available mAbs and subjective identification techniques. The speciation of fungal hyphae and fragments requires species-specific mAbs which, up until now, have not been available for the majority of medically important fungi other than for *Stachybotrys chartarum* (Schmechel et al., submitted for publication). Although our study was based on a cross-reactive mAb, it clearly demonstrates the potential and practicality of the HIA in combination with double immunostaining. As further species-specific mAbs become available, our approach will enable the differentiation of wild-type fungi in air samples, in addition to providing informative patient-specific exposure profiles, which may eventually lead to instructive environment-specific exposure-disease patterns in epidemiological studies.

Understanding the clinical significance of exposure to airborne fungi is well recognized. However, progress has been limited by problems associated with present methodologies (Rogers, 2003). Currently, allergen extracts used for in vivo and in vitro diagnostics lack standardization, are restricted to only a few major fungal species and research based on them is often difficult to reproduce. Furthermore, extensive cross reactivity exists between fungal genera (Horner et al., 1995), leading to uncertainties in the identification of species to which exposure and hypersensitivity is occurring. Double immunostaining based on the HIA has the potential to identify fungal species in personal air samples without the need for any fungal extracts, in addition to simultaneously demonstrating allergic sensitization to individual fungi for each patient. It thus combines environmental with serological monitoring on a patient-specific basis and may well become a significant immunodiagnostic tool ultimately contributing to better patient management and the characterization of adverse health effects due to fungal aerosols.

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