

Demonstration of aeroallergenicity of fungal hyphae and hyphal fragments among allergic rhinitis patients using a novel immunostaining technique

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Background

More than 80 species of fungi are suspected of inducing immunoglobulin E (IgE)-mediated hypersensitivity. Exposure to airborne fungal conidia has been linked to the respiratory symptoms in individuals with fungal allergy; however, the contribution of airborne fungal hyphae and hyphal fragments to allergic diseases is poorly understood.

Objective

We sought to investigate the expression of allergens from airborne fungal hyphae and hyphal fragments using the halogen immunoassay, which uses patients' serum IgE to immunostain immobilized allergens extracted from individual fungal particles.

Materials and methods

Airborne fungi were collected from the nasal cavities of 25 patients and 10 controls using the refined nasal wash technique, fixed on mixed cellulose ester protein-binding membranes, incubated overnight in a humid chamber to promote the germination of conidia, and immunostained with the participants own serum IgE. The samples were examined by means of light microscopy, and positively immunostained fungal particles were classified and counted.

Results

All samples contained fungal particles that expressed soluble allergens and were significantly higher in concentration than counts of conidia of individual well-characterized allergenic genera ($P < 0.05$). Resultant immunostaining of fungal hyphae was heterogeneous, and ~27% of all hyphae expressed detectable allergens compared with nonstained hyphae ($P < 0.05$).

Conclusion

This study conclusively demonstrates that fungal hyphae and fragments are underestimated sources of aeroallergens because positively immunostained hyphal fragments were detected in all samples and the number of the detected fungal hyphae in any of the individual protein-binding membranes was significantly higher than the conidial counts in any of the commonly recognized aeroallergenic species.

Keywords:

fungal aeroallergenicity, fungal fragments, fungal hyphae, halogen immunoassay, immunostaining

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Introduction

Fungi are ubiquitous and commonly grow as saprophytes on nonliving organic material or as invasive pathogens in living tissue. They are principally dispersed as sexual spores or asexual conidia, which are common components of the atmospheric aerospora. Each of these agents has distinctive morphologic features that facilitate the recognition of the genera or species. Fungal hyphae are also aerosolized in large numbers but lack sufficient morphologic characteristics to be taxonomically identified [1,2].

More than 80 species of fungi are suspected of inducing immunoglobulin E (IgE)-mediated hypersensitivity [3]. The most commonly studied fungal species are *Alternaria alternata*, *Aspergillus fumigatus*, *Cladosporium herbarum*, and *Epicoccum purpurascens*, which are prevalent aeroallergen sources throughout

the world, and allergy toward them is a risk factor for allergic rhinitis [4], asthma [5], and even death [6].

Exposure to mold occurs both indoors and outdoors and can vary between individuals and on the basis of geographical location, time, and airflow [7]. The incidence of respiratory fungal allergic disease varies geographically, although it is estimated that 6% of the general population is atopic to fungi [8].

The association between personal exposure to airborne fungi and the manifestation of respiratory disease is complex. In epidemiologic studies, exposure to airborne fungal conidia has been linked to the respiratory symptoms in patients with fungal allergy [9].

The contribution of aerosolized dematiaceous hyphal fragments to the troposphere was first described

after Charles A. Lindbergh's bioaerosol collection expeditions over the Arctic and Atlantic Ocean in 1933 [10]. Ensuing studies identified hyphal fragments as common atmospheric bioaerosols at numerous locations throughout the world [11–14]. Hyphal fragments vary in size (7–100 μm) [12,13] and are characterized in terms of their wall thickness, melanization, septation, and conidiophore features [13,15,16]. Various immunostimulatory macromolecules including antigens [17], allergens [15], mycotoxins [18,19], and (1–3)- β -D-glucans [20,21] have been detected in fungal fragments.

Previous investigations have seldom included the measurement of airborne fungal hyphae and fungal fragments, which might additionally function as aeroallergen sources. Fungal fragments, including airborne hyphae, have been shown to become airborne at significantly higher concentrations compared with conidia in simulated aerosolization experiments [17], and the incorporation of hyphal counts with conidial counts in epidemiologic investigations improved the association with asthma severity [22].

Conventional isolation of the fungal agents by culture, followed by their identification, was used to identify any pathogen to establish etiological infectious disease [23,24]; however, the identification of dematiaceous fungi is somewhat difficult because of their special growth conditions, morphological differences, and the need for correct interpretation of their morphological features. To obtain necessary conidiation, a week or more may be required for incubation of cultures and appropriate sporulation medium should be used [25].

Fungal cultures alone cannot distinguish between colonization and true pathogenicity. Using direct microscopic visualization of samples for identification of the infectious agent is particularly useful when cultures cannot be made or the infectious agent is slow growing [26,27].

Fungal allergic disease can be diagnosed by the in-vivo skin prick test (SPT), by in-vitro detection of allergen-specific serum IgE antibodies (RAST; Pharmacia UniCap assay, CAP, Phadia US Inc. 4169 Commercial Avenue, Portage, Michigan 49002, USA), and in some cases by provocative challenge tests [28]. The most frequently used diagnostic test is the SPT, which uses commercial fungal extracts [29,30]. SPTs are popular as they are less expensive, more convenient, and are generally regarded as being more sensitive. However, there is a drawback as regards the SPT extracts. It is well recognized that such extracts are heterogeneous in allergen content and vary between manufacturers,

batches, and strains [31], and there is probably ongoing degradation of some allergenic components during storage [32]. Another drawback is that within the community, the median wheal size for fungal allergy lies close to the cutoff value conventionally used to discriminate between positive and negative responses [33].

In contrast, in conventional in-vitro assays, such as the CAP assay, the allergens are more stable as they are bound to the solid phase through functional protein groups. However, because of the fact that fungal extracts contain a larger proportion of carbohydrate-associated allergens [32] rather than other common allergens, and as such, carbohydrate or glycoprotein allergens are less likely to be covalently coupled to the solid phase used in CAP assays [34], a slower rate of fungal allergen standardization, compared with other aeroallergen sources [29], is observed.

Typically, a few fungal extracts are available for SPT or in-vitro testing, and it is inferred that these represent the fungal species to which exposure occurs. Further, the lack of concordance between in-vivo and in-vitro fungal diagnostic tests has added to the clinical uncertainty about the accuracy and effectiveness of current fungal allergen diagnostic systems [35,36].

Collection of wild-type fungi from a patient's airway and directly demonstrating allergic sensitization to the same fungal particles has been a technically formidable task [37]. The recently developed halogen immunoassay (HIA) enabled simultaneous visualization of individual fungal particles collected together with their expressed antigens following their immunostaining with the atopic human IgE antibody [38].

HIA is a new approach to the identification of aerosolized fungal particles and the demonstration of allergy toward them. It combines environmental and serological monitoring on a patient-specific basis. HIA as an in-vitro diagnostic technique to detect fungal-specific IgE in human serum correlates well with the conventional allergy diagnostic systems of CAP and, to a lesser extent, with SPT. It represents a new approach to the detection of sensitization to fungal allergens, which may have significant clinical advantages as allergens detected by HIA are analogous to those released during natural exposure. This potentially helps avoid many of the confounding factors that limit both SPT and the in-vitro tests, which depend on retaining the stability of allergens over prolonged periods of storage [39].

This study is an attempt to determine the extent to which airborne fungal hyphal fragments function as aeroallergen sources using the recently described HIA.

Materials and methods

Study population

A total of 25 patients (patient group) with a documented positive clinical history of asthma or allergy-associated nasal symptoms, such as rhinorrhea, nasal obstruction, and/or nasal itching, who had at least one positive SPT response with a wheal diameter of 3 mm or greater when tested with a panel of common inhalant allergens and with a total serum IgE level of more than 100 IU/ml (atopic) were randomly selected from the Fayoum University Outpatient Clinic.

None of the patients received intranasal corticosteroids, antihistamines, antileukotrienes, or oral and intranasal decongestants within 2 weeks before the study, and none of the patients received oral and/or intramuscular corticosteroids within 4 weeks before the study.

A total of 10 randomly selected participants having no allergy-associated symptoms, with negative responses to all common inhalant allergens on SPT (wheal diameter less than 3 mm when tested with a panel of common inhalant allergens), and with a serum total IgE level of less than 100 IU/ml (nonatopic) were included in the study and served as negative controls (control group).

The research ethics committee approved the study protocol and the participants gave written informed consent following a full explanation of the study.

Collection of mucus

A simple noninvasive procedure to obtain as much mucus as possible for testing according to the method by Ponikau [40] was adopted in which two puffs of phenylephrine hydrochloride (1%) were sprayed into each nostril to produce vasoconstriction. The spray also increases the nasal lumen diameter and consequently the yield from nasal lavage. After ~2 min, each nostril was flushed with 20 ml of sterile saline using a sterile syringe with a sterile curved blunt needle. The patient was asked to take a deep inspiratory breath and holds his/her before the injection of saline. The patient then asked to forcefully exhale through the nose during the flushing. The contents from the forceful exhalation were collected in a sterile pan.

The collected fluid was transferred into centrifuge tubes and sent directly to the mycology laboratory where the specimens were processed under a laminar flow hood to prevent contamination. One vial (10 ml) of sterile dithiothreitol was diluted with 90 ml of sterile water. The collected specimen was suspended in an equal volume of diluted dithiothreitol and vortexed

for 30 s. The mixture was allowed to stand at room temperature for 15 min while the dithiothreitol broke apart the disulfide bonds, thus liquefying the mucus. The mixture was then centrifuged at 3000 g in a 50-ml tube for 10 min. The supernatant was discarded, and the sediment was vortexed for 30 s. One-half milliliter of the prepared sediment was applied onto a mixed cellulose ester protein-binding membrane (PBM; 0.8 µm pore size; Millipore Corporation, Bridgewater, Massachusetts, USA).

Halogen immunostaining of the collected mucus

The PBMs were placed in a humid chamber overnight to enable conidia germination. Impacted fungal particles were permanently laminated onto the mixed cellulose ester PBM by overlaying the sample with an optically clear adhesive/glass coverslip, followed by incubation in borate buffer (pH 8.2) for 4 h to enable allergens to elute and bind to the PBM in close proximity to the fungal particle. Membranes were blocked in 5% skimmed milk (SM) in PBS (pH 7.4) for 45 min and then incubated overnight at 4°C with individual patients' sera (dilution 1 : 3, v/v-100 µl patient sera) in 2% SM-PBS-0.05% Tween 20 (Tw). After incubation with the primary antibody, the membranes were rinsed three times in PBS-Tw and incubated for 1.5 h with biotinylated goat anti-human IgE antibodies (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland, USA) diluted to 1 : 500 in 2% SM-PBS-Tw. This was followed by incubation of the membranes for 1.5 h with the ExtrAvidin alkaline phosphatase conjugate (Sigma Chemical Co., St Louis, Missouri, USA) diluted to 1 : 1000 in 2% SM-PBS-Tw. For immunostaining, all samples were incubated with the alkaline phosphatase substrate NBT_BCIP (Pierce Chemical Co., Rockford, Illinois, USA). Staining was then monitored for 20 min periodically until an optimum purple precipitate was obtained. Positively immunostained fungal particles show visible immunostaining of the allergen that is bound to the PBM in close proximity to the conidia or hyphae. The entire PBM was examined at a magnification of ×200 by standard light microscopy [38]. Microscopic observation was carried out on an Image Analysis System (Olympia ver.2.0, Shinjuku Monolith, 3-1 Nishi-Shinjuku 2-chome, Shinjuku-ku, Tokyo 163-0914, Japan).

Each negative control PBM was processed in parallel by substituting patient serum (study group) with [1] negative control (nonatopic) serum, biotinylated goat anti-human IgE, and ExtrAvidin alkaline phosphatase conjugate and alkaline phosphatase substrate NBT_BCIP [2] biotinylated goat anti-human IgE, and

ExtrAvidin alkaline phosphatase conjugate and alkaline phosphatase substrate NBT_BCIP [3] ExtrAvidin alkaline phosphatase conjugate and alkaline phosphatase substrate NBT_BCIP.

Statistical analysis

Differences between the proportion of immunostained and nonimmunostained hyphae, in addition to the total number of fungal conidia and fungal hyphae, were analyzed for significance using the nonparametric Mann–Whitney *U*-test (Analyse-It for Microsoft Excel, Version 1.68; Analyse-It Software Ltd, Leeds, UK). The criterion for significance for all analyses was a *P*-value of less than 0.05 except otherwise noted. All data are expressed as medians and 25th and 75th percentiles.

Results

Fungal spores, conidia, and hyphae were detected in all of the 25 studied samples (patient group), and all PBMs showed detectable levels of antigens on or eluted from the fungal spores, conidia, and hyphae.

The negative control PBM (control group) did not show fungal spores, conidia, or hyphae, or any detectable levels of immunostaining that could be recorded.

Fungal hyphae and fragments

The total number of detected fungal hyphae and hyphal fragments was 149, and ~27% of all hyphae collected on the PBMs showed resultant immunostaining, which was significantly lower ($P < 0.05$) than the proportion of nonstained hyphae.

The detected hyphae varied markedly in size (5–100 μm), shape, color, and hyphal septation.

Resultant immunostaining was heterogeneous and localized primarily to the outer margins of the hyphal tips, the septal junctions, and around the entire fragment (Fig. 1b and c)

The number of detected fungal hyphae in any of the individual PBMs was significantly higher than the conidial counts of *Alternaria* spp. ($P < 0.05$), *Aspergillus* spp. ($P < 0.05$), and *Cladosporium* spp. ($P < 0.05$).

Fungal conidia and spores

Fungal conidia and spores were present in all examined samples, and the total number of detected fungal conidia and spores, whether germinated or not, was 53. The predominant fungal genera

included *Aspergillus*, *Penicillium*, and other genera (producing unicellular colorless conidia called moniliaceous amerspores), as well as *Cladosporium*, ascospore-producing genera, *Fusarium*, *Epicoccum*, and *Alternaria*.

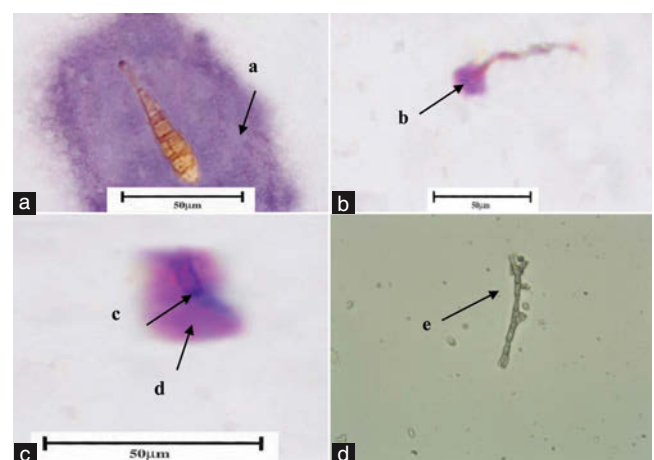
Positive immunostaining was observed in the study population for a wide diversity of fungal spores: 64% (16/25), 24% (6/25), and 16% (4/25) of study participants showed positive immunostaining for moniliaceous amerspores and spores from *Cladosporium* and *Alternaria* spp., respectively.

A lower prevalence of positive immunostaining was also observed among the study population for other fungi, in particular, *Pithomyces* (8%), *Curvularia* (4%), *Bipolaris* (4%), fungi producing ascospores (4%), and *Stachybotrys* (4%).

Resultant immunostaining varied with each individual fungal genus. Moniliaceous amerspore immunostaining was localized around the entire conidia or around the tips of emerged *Cladosporium* hyphae.

Immunostaining of the conidia of species belonging to the family Pleosporales, such as *Alternaria*, *Pithomyces*, and *Bipolaris*, was observed at the beak of *Alternaria* conidia, septal junctions, basal regions, and the outer periphery of the conidia (Fig. 1a).

Fig. 1



(a) Immunostaining of fungi was primarily localized around the outer periphery of multicellular obclavate (25 × 9- μm diameter), brown, regulose conidia with several transverse septa close to each other; longitudinal septa; a short cylindrical beak; and a single scar at the tip. These microscopic characteristics in combination with morphological characteristics confirmed the identification of *Alternaria alternata*. (b) Immunostaining was restricted to the region around the hyphal tips of *Drechslera* spp., which has been distinguished by zig-zag shaped hyphae. (c) In the case of unidentified fungi, septal junctions and all hyphal fragments were surrounded by immune stain.

It is worth mentioning that many other fungal spores that remain unidentified also released allergens that bound human serum IgE and showed positive immunostaining that was heterogeneous and restricted to the septal junctions or around the entire particle.

Discussion

Horner *et al.* [3] estimated that of the 69 000 fungal species described to date, [41] only about 80 species have ever been identified as sources of allergens associated with allergic respiratory diseases mediated by IgE hypersensitivity. Studies on the aerobiology of allergenic fungi usually enumerate 10–20 of the more common genera, whereas the diagnosis of fungal allergy is usually made on the basis of responses to three or four species. This pragmatic approach reflects both the enormous diversity of fungi and the confounders of the diagnostic processes resulting from the lack of standardization, the low stability of extracts, and the variability of source materials [15].

Although the role of aerosolized fungal hyphae and fungal fragments in exacerbating asthma and allergy has not been well studied, preliminary investigations have demonstrated that airborne wild-type fungal hyphae and hyphal fragments express detectable quantities of allergen and can be significantly greater in concentration than the conidia of any single species in indoor residential environments; however, the enumeration and identification of airborne fungal hyphae and fragments by nonviable methods has remained subjective and imprecise [15,17].

The extent to which size influences the release of an allergen from fragments of hyphae has not been studied in detail; cenocytic or nonseptate hyphae have been shown to have the largest critical fragment size, whereas dematiaceous septate hyphal varieties, including *Alternaria* and *Penicillium* spp., have significantly smaller critical fragment sizes [42]. This suggests that smaller-sized dematiaceous fragments might be more capable of releasing greater quantities of allergen compared with cenocytic varieties [15].

This study conclusively demonstrates that fungal hyphae and fragments are underestimated sources of aeroallergens because positively immunostained hyphal fragments were detected in all [24] samples and the number of detected fungal hyphae in any of the individual PBMs was significantly higher than the conidial counts of *Alternaria* spp., *Aspergillus* spp., and *Cladosporium* spp., which are well recognized allergen sources.

Fragmentation, however, was not only restricted to hyphae, and several examples of fragmented conidia were observed. All fragmented conidia were restricted to the genera *Curvularia* and *Exserohilum* and shared similar morphologic characteristics, including conidial septation. Allergen immunostaining was confined to the area surrounding the site of conidial fragmentation and was often in higher concentrations compared with that seen in intact conidia belonging to the same genera.

Our results demonstrated that the proportion of nonstained hyphae and fragments was approximately three times greater than that of positively immunostained hyphae. The interpretation of this variation is relatively unclear, although the amount of allergen released from a hyphal fragment might be a function of the critical fragment size, which is the minimum size to which a fungal fragment remains viable; it is also worth mentioning that particles smaller than 2.5 μm collected on the PBM were impossible to accurately identify by means of standard light microscopy, and it is possible that smaller hyphal and conidial fragments containing allergens remained undetected.

As regards HIA, although it was successfully utilized in environmental and epidemiologic airborne fungal studies, to our knowledge this is one of the first studies that utilized this technique on a clinical basis, acquiring the study samples from the nasal mucous of individual patients (following the maximum mucous collection technique by Ponikau) and not just the surrounding atmosphere and using the individuals' own serum for immunodetection of fungal antigens, not using the well known atopic pool of serum from patients known to have fungal atopy.

In this study, HIA was proven to enable visualization of the morphology of particulates, as well as staining of eluted antigens, and thus speciation on the basis of immunostaining combined with morphology was feasible for conidia, although the detected hyphae and hyphal fragments could be morphologically characterized by a number of features, including hyphal septation and the presence of melanin; however, further identification on the basis of taxonomic criteria involved uncertainty and without species-specific sera cannot currently be used to speciate hyphae. Direct microscopy is probably an additional diagnostic tool; however, it cannot totally replace conventional methods [25] or challenge the novel immunostaining techniques. This finding was observed in our study as particles collected on the PBM were not accurately identifiable using standard light microscopy (Fig. 1d).

The results of this study suggest a much more diverse range of fungal sources of allergens, highlighting

the fungal specification toward immunostaining, and demonstrate that fungal portions as a sources of aeroallergens were in the following order: Fungal hyphal particles > fungal hyphae > fungal conidia. This provides a new paradigm of natural exposure, in which a substantial proportion of the airborne fungal biomass rather than a limited group of genera contributes to the aeroallergen load. The interpretation of personal exposure and sensitization to fungal allergens has, to date, remained restricted to the inhalation of fungal conidia belonging to a select number of species that have been established in bioaerosol studies to be abundant in most geographic locations. Our results independently demonstrate, using a novel immunodiagnostic technique, that airborne fungal hyphae, hyphal fragments, and fragmented conidia express detectable levels of allergens and contribute to personal exposure to fungal allergens. Finally, the aim of this study was to acquaint clinicians and microbiologists with this novel method available for detecting allergic fungal agents by fungal allergic sensitization.

Acknowledgements

Conflicts of interest

None declared.

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