Fungal fragments and undocumented conidia function as new aeroallergen sources

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Background: More than 100 genera of fungal conidia are currently recognized as sources of allergens. The contribution of other fungal genera plus airborne fungal hyphae and fragmented conidia to allergic diseases is poorly understood.
Objective: We sought to investigate the expression of allergens from airborne wild-type fungi using the Halogen immunoassay, which uses allergic serum IgE to immunostain immobilized allergens extracted from individual fungal particles.
Methods: Airborne fungi were collected onto mixed cellulose ester protein–binding membranes for 2.5 hours with volumetric air pumps. Collected fungi were incubated overnight in a humid chamber to promote the germination of conidia. The membranes were laminated with an adhesive cover slip and immunostained with an *Alternaria* species–sensitive serum IgE pool. The samples were examined by means of light microscopy, and positively immunostained fungal particles were classified and counted.
Results: All air samples contained fungal hyphae that expressed soluble allergens and were significantly higher in concentration than counts of conidia of individual well-characterized allergenic genera ($P < .05$). Resultant immunostaining of fungal hyphae was heterogeneous, and approximately 25% of all hyphae expressed detectable allergen compared with nonstained hyphae ($P < .05$). Fungal conidia of 10 genera that were previously uncharacterized as allergen sources were shown to demonstrate IgE binding to expressed antigens and accounted for 8% of the total airborne conidia count.
Conclusions: Our analysis of wild-type fungi collected indoors presents a new paradigm of natural fungal exposure, which, in addition to commonly recognized species, implicates airborne hyphae, fragmented conidia, and the conidia of a much more diverse range of genera as airborne allergens. (J Allergy Clin Immunol 2005;115:1043-8.)

Key words: *Allergen, Alternaria species, antigen, conidia, fragment, fungal, hyphae, germination, immunoassay, mold*

Abbreviations used
HIA: Halogen immunoassay
IOM: Institute of Occupational Medicine
MPBM: Mixed cellulose ester protein–binding membrane
PAS: Personal volumetric air sampler

Fungi are ubiquitous throughout the environment 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. These agents each have 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. Counts of the airborne conidia fluctuate widely in indoor and outdoor environments, with time, and between different geographic regions and climatic conditions. The concentrations can range from 0 to more than 100,000 colony-forming units per cubic meter of air. The most frequent taxa are *Cladosporium*, *Penicillium*, *Aspergillus*, *Alternaria*, and *Aureobasidium* species.[1,2]

Horner et al[3] estimated that of the 69,000 fungal species described to date,[4] only about 80 species have ever been identified as sources of allergens associated with allergic respiratory diseases mediated by IgE hypersensitivity. Studies of the aerobiology of allergenic fungi usually enumerate 10 to 20 of the more common genera, whereas the diagnosis of fungal allergy is usually made on the basis of responses to 3 or 4 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.

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 symptoms of seasonal rhinitis,[5] asthma,[6] and even death[7] in subjects with fungal allergy. This paradigm has been supported by bronchial provocation[8] and longitudinal community[6] studies. However, these investigations have seldom included the measurement of other fungal propagules,

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including airborne hyphae, that might additionally function as aeroallergen sources. Fungal fragments, including airborne hyphae, have been shown to become airborne at significantly higher concentrations than conidia in simulated aerosolization experiments,[9] and the incorporation of hyphal counts with those of conidia in epidemiologic investigations improved the association with asthma severity.[10] To determine the extent to which airborne fungal hyphal fragments and other fungal species function as aeroallergen sources, we collected air samples from a well-ventilated indoor environment and detected the different sources of environmental airborne fungal antigens by using the recently described Halogen immunoassay (HIA).[11,12]

METHODS

Personal air sampling

Personal volumetric air samplers (PASs), which are extensively used in occupational health settings, were used for the current study. The PASs consisted of an Institute of Occupational Medicine (IOM) sampling head (SKC Ltd, Dorset, United Kingdom)[13] connected to a diaphragm pump providing a constant 2.0 L · min^{-1} air flow through a mixed cellulose ester protein–binding membrane (MPBM). The IOM sampling head was sterilized and fitted with a 0.8-μm pore size MPBM (Millipore Corp, Bedford, Mass) for use in the HIA (Woolcock Institute of Medical Research, Sydney, Australia). An indoor residential environment located in Sydney, Australia (34°0′S 151°0'E) was sampled daily over a 21-day period during spring (August-September) for a period of 150 minutes on each day. The IOM sampling heads were placed with the MPBM face on a vertical plane in a location with ample natural ventilation. The collection of air samples coincided with a daily mean temperature of 18.5°C and a mean relative humidity of 46% (Bureau of Meteorology, New South Wales, Australia). Sampling was not conducted if there had been rainfall in the previous 24 hours. Before and after collection, the flow rate of the PAS was remeasured to ensure that a constant 2.0 L · min^{-1} had been maintained.

Human serum samples

Human sera from 30 subjects highly allergic to *Alternaria* species and other fungi were collected and pooled. The diagnosis was based on a documented positive clinical history of asthma or allergy specifically caused by mold, which was determined on the basis of a positive epicutaneous skin prick test response with a wheal diameter of 3 mm or greater. In addition, specific IgE was detected with Pharmacia UniCAP (Pharmacia, Uppsala, Sweden) to a panel of fungal allergens. After collection, all samples were stored in aliquots for future use at −70°C. Pooled serum IgE from 10 subjects with negative skin prick test responses to fungi but sensitized to other nonfungal allergens was included in the study and used as a negative control.

Immunostaining of environmental samples

The MPBM was removed from the IOM sampling head, placed in a humid chamber overnight to enable conidia germination, and immunostained with the HIA as described previously.[11,12] Briefly, in the HIA MPBMs were laminated with an adhesive cover slip and immersed in borate buffer (pH 8.2) for 4 hours to enable allergens and other macromolecules to elute and bind to the MPBM in close proximity to the conidia and hyphae. Membranes were blocked in 1% BSA in PBS and 0.05% Tween 20 (BSA-PBS-Tween 20) for 45 minutes and then incubated overnight at 4°C with pooled human *Alternaria* species–positive sera diluted 1:3 in BSA-PBS-Tween 20. After the primary antibody incubation, the membranes were washed and incubated for 1.5 hours with biotinylated goat antihuman IgE (Kirkegaard and Perry Laboratories, Gaithersburg, Md) diluted 1:500 in BSA-PBS-Tween 20; this was followed by incubation for 1.5 hours with ExtrAvidin alkaline phosphatase conjugate (Sigma Chemical Co, St Louis, Mo) diluted 1:1000 in BSA-PBS-Tween 20 and developed with NBT/BCIP substrate (Pierce Chemical Co, Rockford, Ill), as described previously.[11,12] Samples were examined at a magnification of 200× by using standard light microscopy. Positively immunostained particles displayed visible purple immunostaining only if the sera used contained IgE antibodies specific to the proteins associated with the particles. The number of nonimmunostained and immunostained hyphae, conidia, and fragmented conidia was counted and taxonomically identified to the genus level. Negative controls consisted of similar environmental samples collected on MPBMs and were probed with either (1) nonatopic human fetal chord sera or (2) pooled adult human sera from 10 subjects with negative skin prick test responses to fungi but sensitized to other nonfungal allergens in place of the pooled human *Alternaria* species–positive sera.

Statistical analysis

Differences between the proportion of immunostained and nonimmunostained hyphae, in addition to the total fungal conidia and fungal hyphal numbers, were analyzed for significance by using the nonparametric Mann-Whitney *U* test (Analyse-It for Microsoft Excel, Version 1.68; Analyse-It Software Ltd, Leeds, United Kingdom). The criterion for significance for all analyses was a *P* value of less than .05. Except otherwise noted, all data are expressed as medians and 25th and 75th percentiles.

RESULTS

Airborne fungal spores, conidia, and hyphae expressed detectable levels of antigen in all personal air samples. Collected fungal hyphae varied markedly in size (5-100 μm), shape, color, and hyphal septation (Fig 1). Resultant immunostaining was heterogeneous and localized primarily to the outer margins of hyphal tips (Fig 1, *A*, *B*, *D*, and *F*), the septal junctions (Fig 1, *C*), and around the entire fragment (Fig 1, *E*) and restricted to the site of conidial fragmentation (Fig 1, *G* and *H*). The proportion of fungal hyphae demonstrating immunostaining is presented in Fig 2. Approximately 25% of all hyphae collected on the MPBM demonstrated resultant immunostaining, which was significantly lower ($P < .05$) than the proportion of nonstained hyphae (Fig 2). Similarly, the total number of conidia and hyphae collected in all personal air samples (Fig 3) showed that fungal hyphae were significantly higher in airborne concentration than the conidia counts belonging to *Alternaria* species ($P < .05$), *Aspergillus-Penicillium* species ($P < .05$), and *Cladosporium* species ($P < .05$).

The expression of allergen and subsequent immunostaining of conidia from well-documented allergenic genera, including *Alternaria*, *Aspergillus-Penicillium*, *Cladosporium*, *Exserohilum*, *Curvularia*, and *Pithomyces*