

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 al³ 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 death⁷ in subjects with fungal allergy. This paradigm has been supported by bronchial provocation⁸ and longitudinal community⁶ 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 $2.0 \text{ L} \cdot \text{min}^{-1}$ air flow through a mixed cellulose ester protein-binding membrane (MPBM). The IOM sampling head was sterilized and fitted with a $0.8\text{-}\mu\text{m}$ 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 ($34^{\circ}0'S$ $151^{\circ}0'E$) 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 $2.0 \text{ L} \cdot \text{min}^{-1}$ 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 $200\times$ 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 non-immunostained 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 ($5\text{--}100 \mu\text{m}$), 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 unstained 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*

(Fig 4), was similar to the patterns of IgE staining described in previous culturally derived studies.¹¹ Resultant immunostaining of wild-type conidia was primarily restricted to the germinated hyphal tips, septal junctions, basal regions, and the outer periphery of the conidia of *Alternaria* (Fig 4, A), *Exserohilum* (Fig 4, E), *Curvularia* (Fig 4, D) and *Pithomyces* (Fig 4, F) species, whereas for unicellular conidia belonging to *Aspergillus-Penicillium* (Fig 4, B) and *Cladosporium* (Fig 4, C) species, immunostaining was localized around the entire conidia. In addition, the conidia of fungal genera, previously uncharacterized as allergen sources, were demonstrated for the first time to function as sources of aeroallergen (Fig 5). The localization of IgE binding to expressed antigen was concentrated around the entire spore for *Amphisphaeria* species (Fig 5, A), Myxomycetes (Fig 5, E), *Spegazzinia* species (Fig 5, G), and Ascomycete cleistothecium of the Erysiphales (Fig 5, I); basal regions of the conidia for *Arthrinium* species (Fig 5, B), *Leptosphaerulina* species (Fig 5, D), and *Sporidesmium* species (Fig 5, H); and around the germinated hyphal tips of *Leptosphaeria* species (Fig 5, C), *Pleospora* species (Fig 5, F), and germinated ascospores belonging to the Xylariaceae (Fig 5, J). The total airborne concentrations for each of these undocumented allergenic genera fluctuated widely between air samples and accounted for approximately 8% of the total conidia collected (data not shown).

DISCUSSION

This study conclusively demonstrates that airborne fungal hyphae commonly function as sources of aeroallergen because positively immunostained hyphae were frequently observed on all indoor air samples. The resultant staining was heterogeneous and primarily localized around the entire length of the hyphae, outer margins of the tips, and septal junctions. It is suspected that allergen expression in the vicinity of these sites is attributable to the processes of separation and shear by environmental factors in addition to the cell walls of the hyphae being significantly thinner in regions of septation and cross walls. Several *in situ* factors, including the age of the mycelium and nutrient limitation, have previously been shown to fragment hyphae by initiating hyphal vacuolation, which weakens the tensile strength and rigidity of the hyphae.^{14,15} 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. The manifestation of allergen was confined to the area surrounding the site of conidial fragmentation, often in higher concentrations compared with that seen in intact conidia belonging to the same genera. It is unknown what environmental processes are involved in causing fragmentation; however, it can be expected that these fragmented conidia that would function as allergen sources were nonviable.

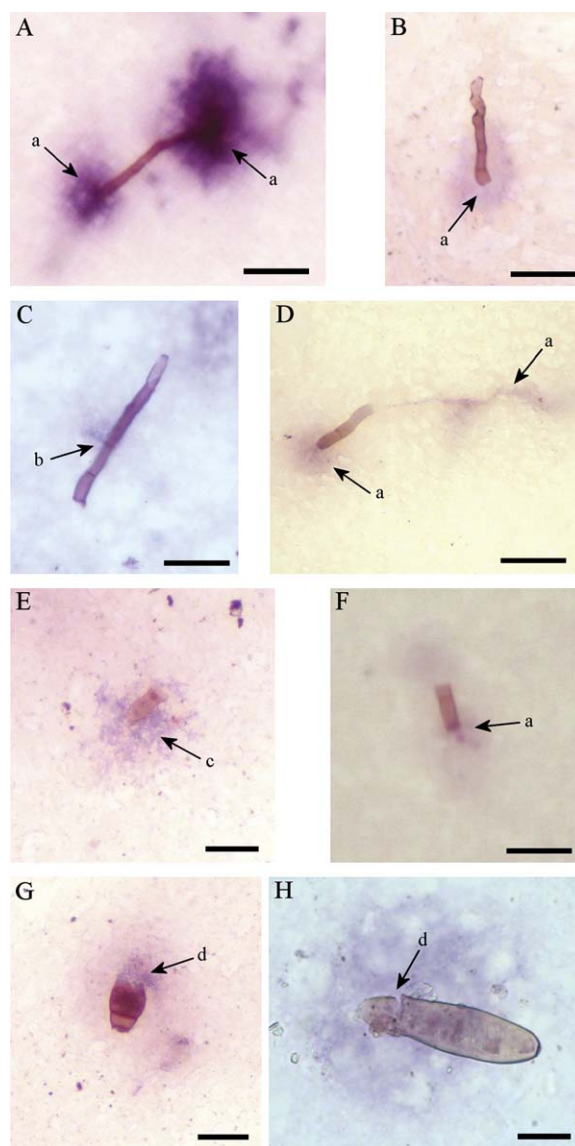


FIG 1. Resultant immunostaining of airborne hyphal fragments confined to the hyphal tips (A, B, D, and F; arrow a), regions of septation or cross-wall linkages (C; arrow b), and around the entire fragment (E; arrow c). Fragmented conidia (G and H) expressed antigen localized at the site of fragmentation (arrow d). Scale bar, 10 μ m (Fig 1, E and F) and 20 μ m (Fig 1, A-D, G, and H).

Our findings demonstrate that the proportion of nonstained hyphae was approximately 4 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. Cenocytic or nonseptate hyphae have been shown to have the largest critical fragment size, whereas dematiaceous septate hyphal varieties, including *Alternaria* and *Penicillium* species, have significantly smaller critical fragment sizes.¹⁶ This suggests that smaller-sized dematiaceous fragments might be more capable of releasing

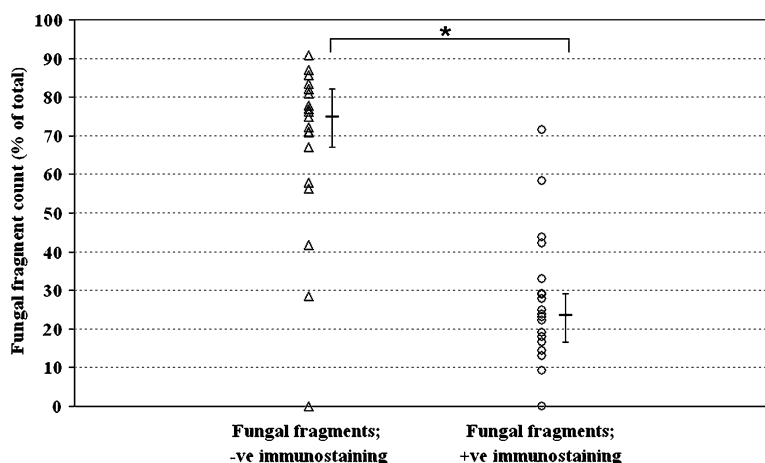


FIG 2. The proportion of airborne hyphal fragments not expressing detectable levels of fungal allergen was significantly higher than positively immunostained fragments ($P < .05$). Results are presented as medians and 25th and 75th percentiles ($n = 21$). Significant differences between the numbers of positively and negatively immunostained airborne hyphal fragments were indicated as follows: $*P < .05$.

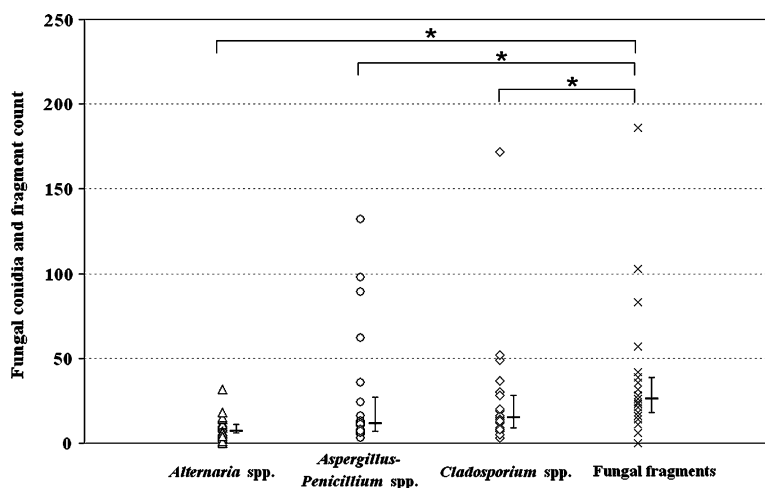


FIG 3. Total fungal counts demonstrated that airborne hyphal fragments were significantly higher in concentration compared with that seen in other fungal genera. Results are presented as medians and 25th and 75th percentiles ($n = 21$). Significant differences between fungal fragment counts and other genera were indicated as follows: $*P < .05$.

greater quantities of allergen than cenocytic varieties. The extent to which viability influences the release of allergen from fragments of hyphae has not been studied in detail. Environmental variables, including the ambient temperature, humidity, solar radiation, and duration that a particle remains airborne might account for this variation in immunostaining by either denaturing or solubilizing antigens; however, this remains anecdotal and requires further investigation. The proportion of hyphae demonstrating immunostaining would also be a function of the specificity of the sera used. In this case a pool of serum from subjects selected for *Alternaria* species skin test sensitivity was used, and individual serum or different serum pools could be expected to have a different spectrum of detection.

Collected airborne hyphae could be morphologically characterized by a number of discernable features, including hyphal septation and the presence of melanin; however, further identification on the basis of taxonomic criteria involves uncertainty. The concentration of total airborne hyphae was significantly higher than the conidia of a number of common and well-characterized individual genera, including *Alternaria*, *Aspergillus*, *Penicillium*, and *Cladosporium*. Aerosolization studies with culturally derived fungal sources also showed fungal fragments outnumbering conidia.⁹ Particles smaller than 2.5 μm collected on the MPBM were unable to be accurately identified by means of standard light microscopy, and it is possible that smaller hyphal and conidial fragments containing allergens were undetected.

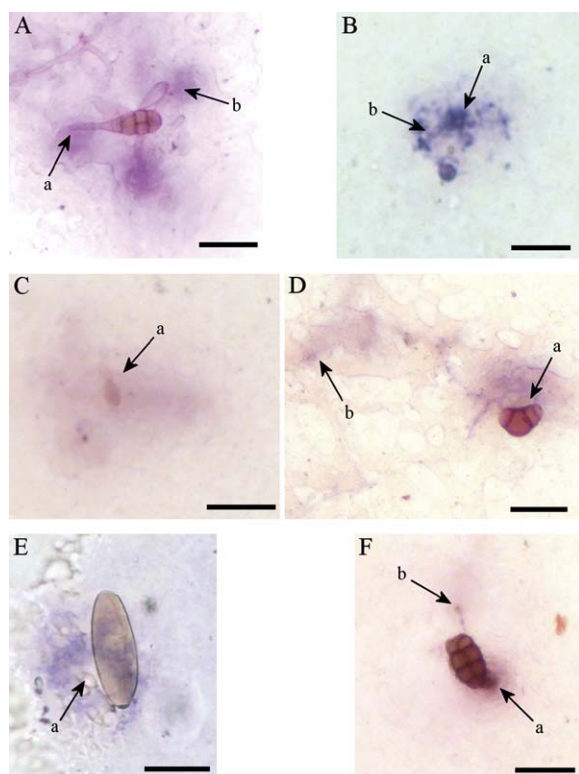


FIG 4. Deuteromycete conidia (arrow *a*) and germinated conidia (arrow *b*) represented by *Alternaria* (A), *Aspergillus-Penicillium* (B), *Cladosporium* (C), *Curvularia* (D), *Exserohilum* (E), and *Pithomyces* species (F) demonstrated IgE binding to expressed antigens. Scale bar, 10 μ m (Fig 4, B) and 20 μ m (Fig 4, A and C-F).

Personal exposure to airborne fungi in indoor and outdoor environments is heterogeneous and has been shown to differ between individuals in experimental studies.¹⁷ The aerosolization of fungal hyphae and conidia is dependent on a number of location-specific atmospheric parameters, including wind velocity, wind direction, and substrate disturbance.⁹ The release of fungal hyphae is additionally dependant on the morphologic characteristics of the species and of the mycelium, as shown in the case of *Aspergillus versicolor* and *Penicillium melinii*.⁹ It could be anticipated that personal exposure to a range of fungal conidia and hyphae would fluctuate independently, as well as widely, between seasons and study locations.

In addition to well-recognized allergen sources, such as *Alternaria*, *Aspergillus-Penicillium*, *Cladosporium*, *Exserohilum*, *Curvularia*, and *Pithomyces* species, a number of other fungal genera (approximately 8% of the total conidia collected) were demonstrated as sources of allergen, as determined by IgE binding to expressed antigen in the HIA. These included conidia and spores belonging to Ascomycetes, Deuteromycetes, and Myxomycetes, all of which had characteristic localization of allergen concentrated around the basal regions of the spores, conidia, cleistothecia, and growing tips of the germinating hyphae. Our findings suggest a much more diverse range of fungi function as sources of allergen. This

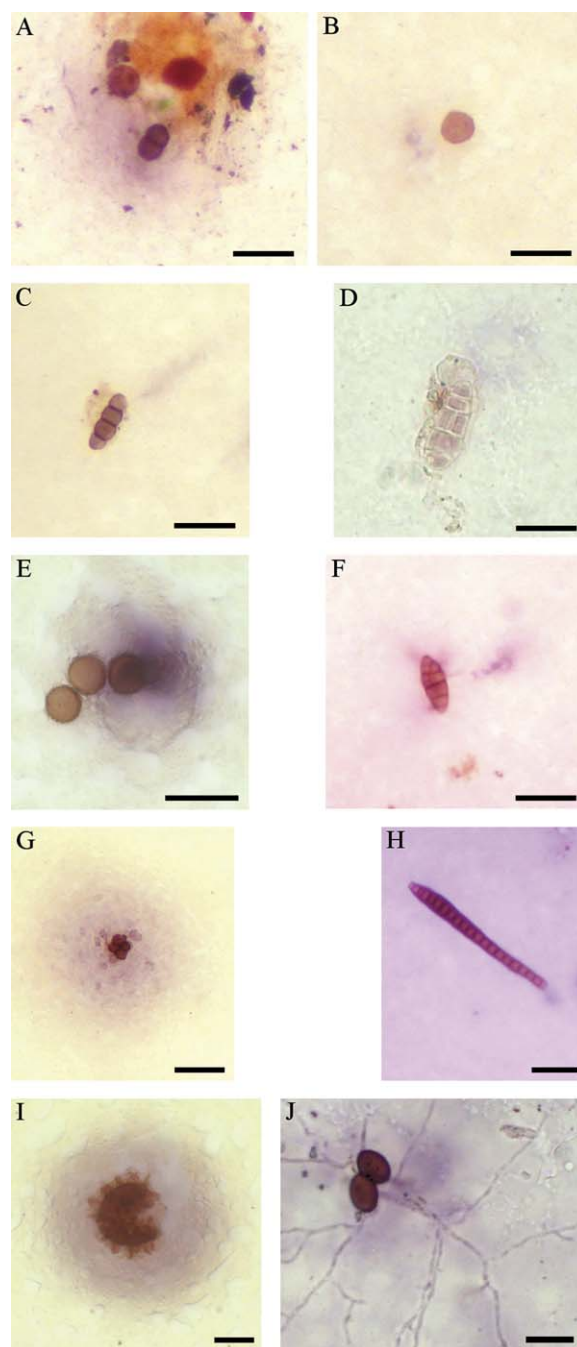


FIG 5. Undocumented fungal genera demonstrated IgE binding to expressed antigens, including *Amphisphaeria* species (A), *Arthrinium* species (B), *Leptosphaeria* species (C), *Leptosphaerulina* species (D), Myxomycete spores (E), *Pleospora* species (F), *Spe-gazzinia* species (G), *Sporidesmium* species (H), Ascomycete cleistothecium of the Erysiphales (I), and germinated ascospores belonging to the Xylariaceae (J). Scale bar, 10 μ m (Fig 5, A and B), 20 μ m (Fig 5, C-H and J) and 50 μ m (Fig 5, I).

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. Although these experiments were

performed with a pooled serum and thus might over-represent the diversity for an individual, the same experiments could be repeated, with individual serum providing a view of individual responses to the environmental fungal load. It is plausible that individuals will recognize allergens from the common species as frequently as they did the less common species because all genera showed a similar strength of immunostaining.

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 and fragmented conidia, as well as fungal genera not previously considered as allergen sources, express detectable levels of allergen and contribute to personal exposure to fungal allergens.

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REFERENCES

1. Burge HA, Pierson DL, Groves TO, Strawn KF, Mishra SK. Dynamics of airborne fungal populations in a large office building. *Curr Microbiol* 2000;40:10-6.
2. Gots RE, Layton NJ, Pirages SW. Indoor health: background levels of fungi. *AIHA J* (Fairfax, Va) 2003;64:427-38.
3. Horner WE, Helbling A, Salvaggio JE, Lehrer SB. Fungal allergens. *Clin Microbiol Rev* 1995;8:161-79.
4. Levetin E. Fungi. In: Burge HA, editor. *Bioaerosols*. Boca Raton (FL): Lewis Publishers; 1995. p. 87-120.
5. Li DW, Kendrick B. Indoor aeromycota in relation to residential characteristics and allergic symptoms. *Mycopathologia* 1995;131:149-57.
6. Downs SH, Mitakakis TZ, Marks GB, Car NG, Belousova EG, Leuppi JD, et al. Clinical importance of *Alternaria* exposure in children. *Am J Respir Crit Care Med* 2001;164:455-9.
7. Black PN, Udy AA, Brodie SM. Sensitivity to fungal allergens is a risk factor for life-threatening asthma. *Allergy* 2000;55:501-4.
8. Licorish K, Novey HS, Kozak P, Fairshier RD, Wilson AF. Role of *Alternaria* and *Penicillium* spores in the pathogenesis of asthma. *J Allergy Clin Immunol* 1985;76:819-25.
9. Gorny RL, Reponen T, Willeke K, Schmechel D, Robine E, Boissier M, et al. Fungal fragments as indoor air biocontaminants. *Appl Environ Microbiol* 2002;68:3522-31.
10. Delfino RJ, Zeiger RS, Seltzer JM, Street DH, Matteucci RM, Anderson PR, et al. The effect of outdoor fungal spore concentrations on daily asthma severity. *Environ Health Perspect* 1997;105:622-35.
11. Green BJ, Mitakakis TZ, Tovey ER. Allergen detection from 11 fungal species before and after germination. *J Allergy Clin Immunol* 2003;111:285-9.
12. Mitakakis TZ, Barnes C, Tovey ER. Spore germination increases allergen release from *Alternaria*. *J Allergy Clin Immunol* 2001;107:388-90.
13. Mark D, Vincent JH. A new personal sampler for airborne total dust in workplaces. *Ann Occup Hyg* 1986;30:89-102.
14. Li ZJ, Bhargava S, Marten MR. Measurements of the fragmentation rate constant imply that the tensile strength of fungal hyphae can change significantly during growth. *Biotechnol Lett* 2002;24:1-7.
15. Paul GC, Kent CA, Thomas CR. Hyphal vacuolation and fragmentation in *Penicillium-chrysogenum*. *Biotechnol Bioeng* 1994;44:655-60.
16. Marfenina OE, Ivanova AE, Zvyagintsev DG. The effect of fragmentation of the mycelium of various yeast species on its viability. *Microbiology* 1994;63:603-6.
17. Mitakakis TZ, Tovey ER, Xuan W, Marks GB. Personal exposure to allergenic pollen and mould spores in inland New South Wales, Australia. *Clin Exp Allergy* 2000;30:1733-9.