

## NOTES

# Detection of Aerosolized *Alternaria alternata* Conidia, Hyphae, and Fragments by Using a Novel Double-Immunostaining Technique

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**A double-immunostaining halogen immunoassay was developed to identify aerosolized conidia, hyphae, and fragments of *Alternaria alternata* by using an anti-*Alternaria* polyclonal antiserum, while, simultaneously, allergy to these components was concurrently determined by using human immunoglobulin E antibodies.**

Fungi are eukaryotic microorganisms that are ubiquitous bioaerosols in the environment. The fungus *Alternaria alternata* is an extensively distributed plant pathogen, and allergy to it is a risk factor for asthma severity (8). The reproductive structures, which characterize this species and aid in the organism's dispersal, include multicellular conidia and septate hyphae. Airborne *A. alternata* conidia have been established in numerous studies to be prevalent in both indoor and outdoor environments (2), although the role of aerosolized hyphae in exacerbating asthma and allergy has not been established. Recent preliminary investigations have demonstrated that airborne wild-type fungal hyphae express detectable quantities of allergen (5) and can be significantly greater in concentration than the conidia of any single species in indoor residential environments (3, 5). Until now, the enumeration and identification of airborne hyphae by nonviable methods has remained subjective and imprecise, and improved objective detection methods for fungal bioaerosol monitoring are required.

Numerous attempts have been made to detect and identify specific fungal components that serve as allergen sources. One such technique has been direct immunostaining with human immunoglobulin E (IgE) of insoluble fungal antigens on the surface of wild-type fungal conidia (9). However, this technique does not permit speciation or detect hyphae. The recent development of monoclonal antibodies specific for some soluble fungal allergens does not provide a basis for speciation either, as these allergens are often shared between fungal species (10). Up until now, there has not been a method that can potentially differentiate between different species of aerosolized hyphae collected during volumetric air sampling that function as allergen sources. In this study, we describe for the first time proof of principle of a novel double-immunostaining methodology based on the halogen immunoassay (HIA), which (i) enables the identification of *A. alternata* conidia, hyphae,

and fragments by immunostaining eluted antigens with a polyclonal antibody (pAb) and (ii) concurrently demonstrates allergy to the same fungal particles by immunostaining with human IgE.

The method is briefly described as follows. Conidia and hyphae belonging to *A. alternata* were double immunostained using the HIA with an anti-*Alternaria* pAb (Woolcock Institute of Medical Research, Sydney, Australia) and a serum IgE pool from *Alternaria*-sensitive subjects (Pharmacia CAP score with specific IgE to *A. alternata* = 60.7 kU<sub>A</sub>/liter). The rabbit antiserum was raised against a crude in-house *Alternaria* extract and was shown in these studies to have a broad specificity for different *Alternaria* components. Enzyme-linked immunosorbent assay analysis using crude fungal extracts showed the sera to have strong binding to this species and weak binding to several other species tested (data not presented). Negative controls consisted of a pool of sera from atopic but *Alternaria*-negative subjects (Pharmacia CAP score with specific IgE to *A. alternata*, <0.35 kU<sub>A</sub>/liter) and rabbit pAb raised against a crude *Lolium perenne* pollen extract.

For the halogen immunoassay, components of *A. alternata*

TABLE 1. Summary of the proportion of ungerminated and germinated *A. alternata* conidium treatments demonstrating double immunostaining with pAb and human IgE

| State of fungal germination <sup>a</sup> | Mean proportion of fungal conidia (range) <sup>b</sup> |                               |
|------------------------------------------|--------------------------------------------------------|-------------------------------|
|                                          | No immunostaining                                      | pAb + IgE immunostaining      |
| Ungerminated conidia                     | 51.7 (48.7–54.7)                                       | 48.3 (45.2–51.3)              |
| Germinated conidia                       | 5.8 (2.7–10.6)                                         | 94.2 (89.3–97.2) <sup>c</sup> |

<sup>a</sup> All *A. alternata*-negative control treatments showed no immunostaining around the ungerminated conidia and germinated conidia and hyphae.

<sup>b</sup> 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, underdevelopment of the precipitating stains may have underrepresented the proportion of conidia demonstrating double immunostaining.

<sup>c</sup> Germinated conidium double-immunostained values, which are significantly higher than those double immunostained in the ungerminated conidium treatment ( $P < 0.0001$ ).

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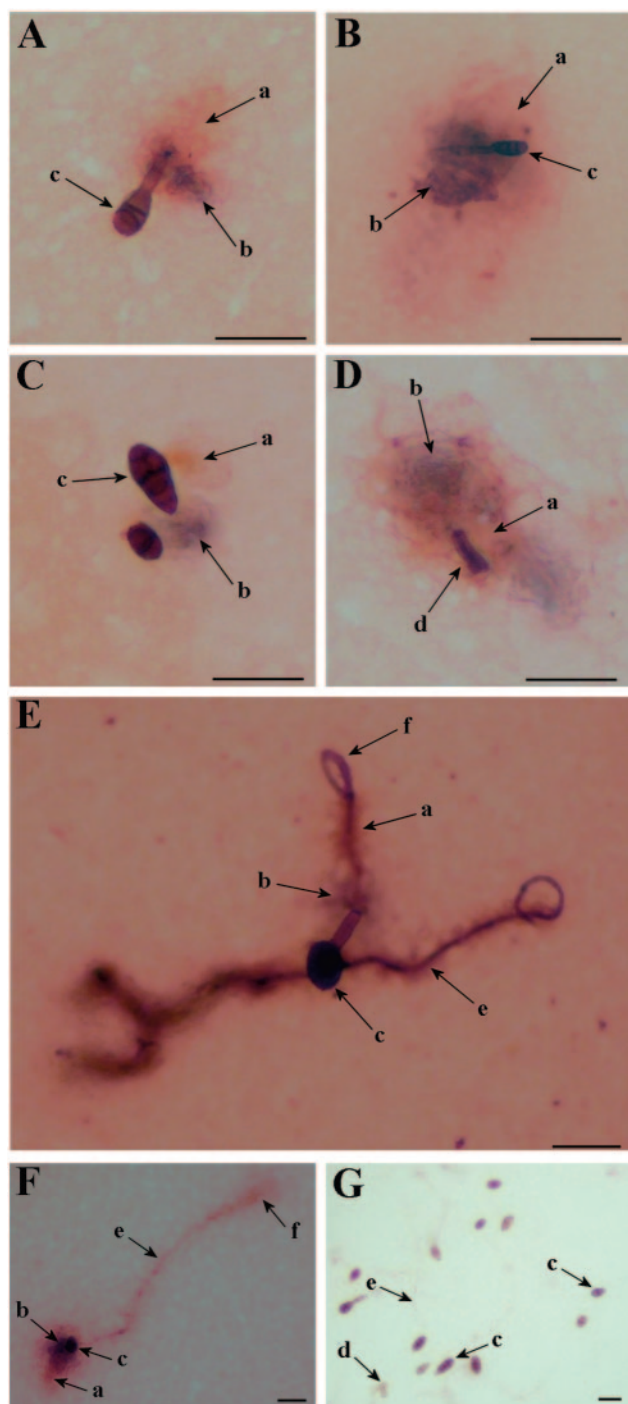


FIG. 1. Resultant double immunostaining of culture-derived *A. alternata* conidia (A to C), an *A. alternata* hyphal fragment (D), and germinated *A. alternata* conidia (E to F), using a pAb raised against a crude *Alternaria* extract (arrow a, red precipitate) and human serum IgE (arrow b, purple precipitate). Immunostaining was confined to (A) basal regions of the conidia (arrow c), (B) around the entire conidia (arrow c), (C) septal junctions of the conidia (arrow c), (D) around the entire fungal hyphal fragment (arrow d), and (E to F) around the hyphae (arrow e) and hyphal tips (arrow f) of germinated conidia (arrow c). (G) Negative controls using a pool of sera from atopic but *Alternaria*-negative subjects (Pharmacia CAP score with specific IgE to *A. alternata*,  $<0.35$  kU<sub>A</sub>/liter) and an in-house rabbit pAb raised against a crude *Lolium perenne* pollen extract showed no localized immunostaining around the conidia (arrow c), hyphal frag-

were aerosolized from culture plates by using an air jet and then collected by vacuum onto a mixed cellulose ester (MCE) protein-binding membrane (0.8- $\mu$ m pore size; Millipore Corporation, Bridgewater, MA) as previously described (4). The collected fungal particles were either germinated under humid conditions for 12 h or were not germinated, before being permanently laminated to the MCE by overlaying them with a glass coverslip that had been precoated with a thin film of optically clear adhesive (4). The laminated samples were immersed in borate buffer (pH 8.2) for 4 h to enable antigens and other macromolecules to elute from the conidia and hyphae and bind to the membrane. Membranes were blocked in 1% bovine serum albumin in phosphate-buffered saline (PBS) for 45 min and then incubated overnight with the positive IgE serum pool diluted 1:3 in 1% bovine serum albumin-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 & Perry Laboratories, Gaithersburg, MD) diluted 1:500. This was followed by an incubation for 1.5 h with the pAb diluted 1:100. The membranes were rinsed and then incubated for 1.5 h with an anti-rabbit IgG-horseradish peroxidase conjugate (Sigma Chemical Co, St Louis, MO) and an ExtrAvidin-alkaline phosphatase conjugate (Sigma Chemical Co, St Louis, MO), each at a titer of 1:1,000, and then rinsed three times. For immunostaining, Vector NovaRED substrate for horseradish peroxidase was prepared as per the manufacturer's instructions (Vector Laboratories, Burlingame, CA) and added to the MCE for approximately 1 h to allow adequate development of the red precipitate. The membrane was then rinsed three times and transferred to a separate staining well containing the alkaline phosphatase substrate nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (Pierce Chemical Co, Rockford, Ill.). Staining was then monitored periodically for up to 20 min until optimum dark blue precipitate development was achieved. Samples were examined at a magnification of  $\times 200$  by using standard light microscopy, and double-immunostained fungal particles were considered positive and counted. A Student's *t* test was performed with Analyse-It software (Analyse-It Software Ltd., Leeds, United Kingdom) to compare mean values for double-immunostained subsets among ungerminated and germinated groups. A difference was considered significant when *P* was  $<0.0001$ .

As shown in Table 1, the results of double immunostaining demonstrated that 48% of ungerminated conidia expressed detectable pAb and IgE immunostaining; however, upon germination this rose to 94% ( $P < 0.0001$ ). Negative controls showed no immunostaining around the outer edges of the ungerminated and germinated conidia or hyphae (Fig. 1G; Table 1). In the controls, there was some purple staining of the interior of the conidia but not the hyphae, which is consistent with the nitroblue tetrazolium functioning as a vital stain of viable spores. These findings are consistent with previous studies, which have demonstrated that the germination of conidia

ments (arrow d), and hyphae (arrow e). The diffuse homogeneous background staining observed in the positive samples (A to F) was absent in the negative controls (G). Scale bar, 25  $\mu$ m.

belonging to *Aspergillus fumigatus* and other fungal genera increases the proportion of conidia releasing detectable allergen (4, 7, 12), which are detected in the periphery of the components. Conidial germination is an important process in this immunoassay as it significantly increases the number of conidia being detected, in addition to improving the detectable thresholds of the immunoassay.

Results from this study also indicate that the localization of immunostaining was heterogeneous between both conidia and the state of germination. The sites of immunostaining associated with ungerminated conidia varied from being restricted to the basal regions (Fig. 1A) and septal junctions (Fig. 1C) to being found around the entire conidia (Fig. 1B). It is unclear whether this represents different sites of antigen or allergen release or is an artifactual effect of the assay, as the antigens have to diffuse from the particle until bound to the membrane. Upon conidial germination, where long hyphae are evident, greater concentrations of double immunostaining were observed. In the germinated conidia, antigen staining by the pAb was associated with the entire hyphae, whereas the IgE immunostaining was confined to the hyphal tips and the sites of conidial germination (Fig. 1E and F). The heterogeneous localization of the resultant immunostaining between conidia and the states of germination confirms our earlier reports investigating other fungal genera (4, 7).

Improvements in technical aspects of the HIA allow improved resolution of the expression of allergens by the conidia and other fungal components, not previously observed (7), and demonstrate for the first time the localized expression of soluble antigens released from *A. alternata* conidia. Previous studies have shown that the release of proteins, glycoproteins, and carbohydrates may be determined by the conidial wall structure (1, 6). Thin-walled conidia have been shown to release allergen quickly, whereas those with thicker walls have been found to contain a rodlet layer (1), which functions to reduce cytoplasmic leakage. These results suggest that the localization of *A. alternata* conidial antigen could be due to variations in the thickness of the conidial wall, especially as immunostaining was localized around thin-walled septal junctions.

The development of the described double-immunostaining technique was initiated as a result of recent observations, which showed that the airborne concentration of fungal hyphae and fragments was higher than that of conidia of any single species (3, 5) and that these fragments released detectable quantities of allergen (5). Fungal fragments are heterogeneous particles, which are unable to be speciated by nonviable morphological characteristics or distinguished from conidia as antigen sources by current techniques. Our findings demonstrate that morphologically indiscernible fragments and hyphae were double immunostained with pAb and IgE (Fig. 1D). This proof-of-principle finding is important as it enables for the first time the identification of whether the fragment is of fungal origin by detecting expressed fungal antigens, in addition to demonstrating IgE binding to allergens eluted from these fragments.

The determination of individual fungal species by immunostaining techniques is dependent on the specificity of the antibodies available. Although several fungal allergen-specific monoclonal antibodies have been produced, these allergens

are produced by multiple fungal species and are not restricted to a single species (10). To our knowledge, only one species-specific antibody, against *Stachybotrys chartarum*, has been described (11). The halogen immunoassay enables visualization of the morphology of particulates as well as staining of eluted antigens, and so speciation on the basis of immunostaining combined with morphology is feasible for conidia but without species-specific sera cannot currently be used to speciate hyphae. This paper provides a proof of concept how this could proceed, and the future availability of different species-specific antibodies will allow this technique to be applied to environmental samples, to further differentiate the airborne fragments, and to provide a comprehensive immunodiagnostic and environmental monitoring technique based not only on conidia but also on hyphae and conidial fragments. This would not be possible by any other current technique.

In conclusion, our study has demonstrated a new approach to the identification of aerosolized fungal particles and demonstrating allergy to them. It combines environmental with serological monitoring on a patient-specific basis and has the potential to be a significant immunodiagnostic tool and contribute to improved characterization of adverse health effects due to fungal aerosols.

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