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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

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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.

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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

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