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Technical note

Detection of allergens from *Alternaria alternata* by gold-conjugated anti-human IgE and field emission scanning electron microscopy

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Abstract

Fungal allergens are present in viable and non-viable conidia, hyphae and fungal fragments. It has been shown that large quantities of allergen are released from conidia during germination. We used a gold immunolabelling technique and field emission scanning electron microscopy to examine the allergen release from *Alternaria alternata* conidia. Immunolabelling was associated with the hyphal tip and amorphous matter associated with the emerging hyphae. Non-specific antibody controls showed no labelling associated with germinating fungi. This suggests that material released from hyphae may be an additional source of fungal allergens.
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1. Introduction

Airborne fungi have a widespread and highly variable occurrence and are among the most common bioaerosols that humans inhale. Interest in measuring exposure to airborne fungi has increased dramatically in recent years due to the complex and diverse associations suspected to occur between such exposure and adverse health effects, notably allergic rhinitis and asthma (Downs et al., 2001), but also 'sick building syndrome' and a range of other disorders, as recently reviewed (Bush et al., 2006).

Some fungal allergens are present in viable and non-viable conidia, hyphae and fungal fragments and it has been shown by halogen immunoassay (HIA) for a number of species that large quantities of allergen are released during germination (Mitakakis et al., 2001; Green et al., 2003). In the HIA technique, aerosol particles are immobilised on a protein-binding membrane and their allergens eluted and adsorbed to the membrane and subsequently immunostained using allergen-specific IgE or monoclonal antibodies. This results in specific labelling of the individual particles carrying the allergens

Abbreviations: HIA, halogen immunoassay; SEM, scanning electron microscopy; MCE, mixed cellulose ester; BSA/PBS Tween:2% bovine serum albumin in phosphate-buffered saline with 0.05% Tween 20.

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(Tovey et al., 2000). HIA of fungal aeroallergen sources results in staining of allergenic conidia, which can be classified based on their morphology, as well as smaller allergenic fungal components, which lack features that allow their visual identification.

The increased production of allergens by conidia during germination (Green et al., 2003), as well as exposure to smaller fungal fragments, may be important factors in the development of fungal allergy and clinical outcomes (Green et al., 2005). However, the current chromogenic-based HIA is limited by the resolution achievable with light and confocal laser scanning microscopy, making fungi with smaller unicellular conidia, hyphae and fragments difficult to resolve. We therefore modified the HIA to enable it to be used with scanning electron microscopy (SEM) to provide greater resolution and magnification of allergen expressed by conidia and fungal components.

2. Materials and methods

2.1. Preparation of germinated *Alternaria alternata* conidia

A. alternata cultures (AL4 isolate, University of Sydney) were grown for 25 days on vegetable juice nutrient agar. Conidia were aerosolised using a gentle air stream, and collected by suction onto a 0.22 μm mixed cellulose ester (MCE) protein-binding filter membrane (Millipore, Bedford, MA, USA) as previously described (Green et al., 2003). The conidia were germinated for 6 h at 25 °C and 98% relative humidity and then fixed in glutaraldehyde vapour for 10 min.

2.2. Atopic and control human sera

Pooled human serum containing anti-fungal human IgE, collected from 5 atopic subjects who were skin-prick test positive (wheal diameter ≥3 mm) to an *Alternaria* allergen preparation (Hollister-Stier, Toronto, Canada), was used as the primary antibody. Serum from a subject who was skin-prick test negative to fungi but positive to house dust mite allergen was used as a negative primary antibody control.

2.3. Immunostaining of fungal allergens

HIA was performed similarly to previous descriptions (Green et al., 2003, 2005) except that samples were not laminated, but were placed directly in 0.2 M borate buffer pH 8.2 for 16 h at 4 °C to enable allergens and other macromolecules to elute and bind in close proximity to the conidia and hyphae on the MCE. Any excess binding capacity of the membranes was then blocked by incubation in 2% bovine serum albumin in phosphate-buffered saline with 0.05% Tween 20 (BSA/PBS Tween) for 2 h. The primary antibody probing was performed using the fungal-positive serum pool, diluted 1/4 in 1% BSA/PBS Tween, with incubation for 16 h at 4 °C. Samples were then washed 3 times in PBS Tween, and incubated with goat anti-human IgE conjugated to 40 nm colloidal gold microspheres (MDM golds, Australia) diluted 1/500 in 1% BSA/PBS Tween for 16 h at 4 °C. Samples were again washed 3 times in PBS Tween and dried in air at 25 °C. Negative control samples were prepared in parallel, using the human serum negative to *A. alternata* as the primary antibody. Further negative controls were performed using no primary antibody at all.

2.4. Field emission scanning electron microscopy (field emission SEM)

A 3 mm^2 specimen was cut from the filter and mounted on an aluminium specimen holder using double-sided carbon tape (ProSciTech Pty. Ltd., Queensland, Australia). The specimen was coated with approximately 5 nm of platinum in a modified Edwards E306A sputter coater (Edwards Vacuum Systems, UK) and viewed with a JEOL JSM-6000F field emission SEM (JEOL Ltd. Tokyo, Japan).

3. Results

Conidia in an early stage of germination were selected, as earlier studies of *A. alternata* had shown intensive staining close to the site of germination (Green et al., 2005) (Fig. 1a). When viewed in the backscattered electron mode, bright particles approximately 40 nm in diameter, the known size of the immunogold reagent used, were observed on the hyphal tip and in a region outside the tip where the filter matrix was covered with amorphous matter probably originating from the emerging hyphae (Fig. 1b). The amorphous matter can be seen in Fig. 1a and b where it has filled the voids in the filter matrix.

In instances where the hyphae had covered larger distances, the bright particles were seen in association with the hyphae and extra cellular material in proximity to the hyphae. Very few bright particles were observed on the membranes away from fungal elements. Negative control samples showed no bright particles associated with germinating fungi.

In additional observations, immunostaining by the 40 nm gold microspheres was visible by light microscopy

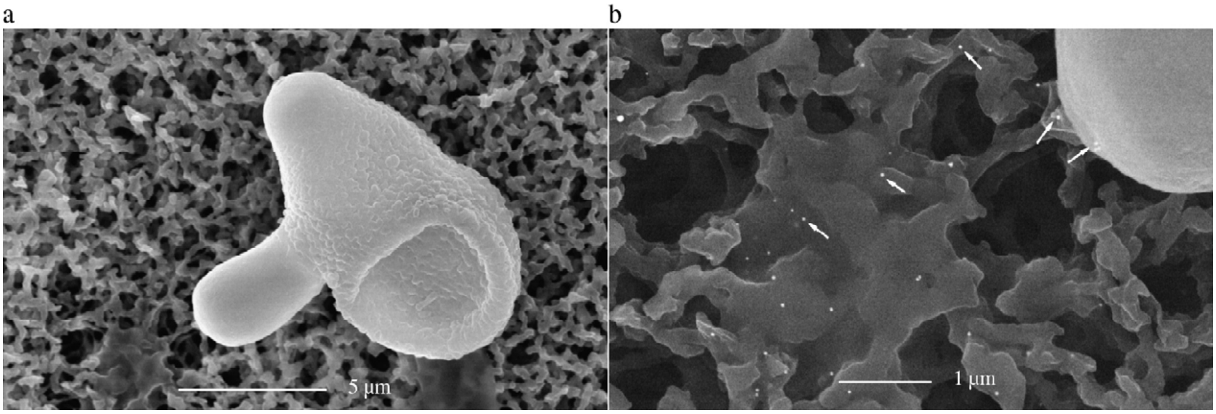

Fig. 1. (a) Germinating *A. alternata* spore viewed with a field-emission SEM in the secondary electron mode. (b) Hyphal tip of germinating spore from Figure 1a and matter outside the tip stained with 40 nm immuno-gold particles. Viewed with a field-emission SEM in the back scattered electron mode. All negative control images showed no immunostaining of spores or germinated hyphae.

on samples prior to sputter-coating. Although this was not as intense as the NBT/BCIP chromogenic staining used in the standard HIA, it was of interest as it can be used as an aid to preparing specimens for electron microscopy.

4. Discussion

These results show that allergenic materials released during the germination of fungal conidia can be visualised at high resolution with a field emission SEM. As far as we are aware, this is the first study to show this. Our previous observations using conventional HIA and these observations using a modified HIA suggest that there are three separate sources of fungal allergens likely to be present in the environment: identifiable intact spores or conidia, non-identifiable fragments of these and fragments of hyphae, and of novel interest, amorphous material released from hyphae that itself contains fungal allergens.

This proof-of-concept study used *A. alternata* as the model species because sera from subjects sensitised to this species was available to us. However, the high resolution that can be obtained with the field emission SEM (0.7 nm) strongly indicates that the technique can be applied to smaller unicellular spores and other smaller particles, including fragments of hyphae and fragmented conidia.

The high electron luminosity and the high resolution provided by the field emission SEM seems to be a requirement for reliable recognition of the immunogold particles under the 5 nm sputter coat of platinum, as attempts with a conventional SEM in the backscattered electron mode were unsuccessful in resolving particles in the same region as those depicted in Fig. 1b (results not shown).

These results demonstrate that the allergens produced by *A. alternata* are contained within material exuding from the hyphae that can be visualised by SEM. While the composition of this extracellular material is not known, it is likely to include polysaccharides and enzymes that are exported during germination (Apoga and Jansson, 2000). There have been few previous studies of fungi and allergens by EM methods; these are mainly confined to observations of their morphology and associations with allergens, including the recent observation of Alt a 1 in the cell walls of conidia and hyphae using transmission electron microscopy by Ibarrola et al. (2004), confirming the observations of Paris et al. (1990) that this allergen accumulated in the culture fluid. Ibarrola et al. (2004) observed intra- and extra-cellular labelling of Alt a 1, indicating that the allergen was excreted, but this was not associated with specific structures as observed in our study using SEM.

Other recent findings also suggest that fungal allergens are present on particles other than intact conidia, including hyphae and fragments of conidia (Green et al., 2005), while others have indicated that fungal antigens can exist on sub-micron aerosol particles (Gorny et al., 2002). Our demonstration of localised IgE binding in areas surrounding hyphal growth, and the association of IgE binding to hyphal exudates, illustrates a mechanism by which highly allergenic fungal material that is not enclosed in spores may be generated.

This is the first time of which we are aware that allergen production associated with the germination of fungal conidia has been visualised using high-resolution field emission SEM and IgE-labelled gold. These observations support a model where fungi may contribute a mixed allergenic bioaerosol including conidia, hyphae and smaller amorphous fragments. Each of these would be deposited at different sites in the airways based on their particle size, and would express allergen in combination with different non-allergenic fungal components, affecting innate and adaptive responses to this exposure.

The techniques that we have described, if used in conjunction with appropriate environmental air sampling techniques, would provide a feasible method of quantifying allergenic particles of fungal origin. In an ideal situation, this would be achieved using allergen-specific monoclonal or polyclonal antibodies, however the use of human serum from sensitised individuals is more readily achievable and also provides clinically relevant information. Further development of these techniques will contribute to the better understanding of the nature of fungal allergen exposure.

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