Detection and diagnosis of fungal allergic sensitisation

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Declaration

I hereby declare that the work presented in this thesis has been carried out by myself and does not incorporate any material previously submitted for another degree in any University. To the best of my knowledge and belief, it does not contain any material previously written or published by another person, except where due reference is made in the text. I am willing to make the thesis available for photocopy and loan if it is accepted for the award of the degree.

B.J. Green

Abstract

Airborne fungi are ubiquitous in the environment and human exposure is inevitable. Such fungi differ greatly in their taxonomic, physical, ecological and pathogenic characteristics. Currently, 69 000 species have been taxonomically classified and more than 80 of these are recognised to be aeroallergen sources. Many strategies have evolved to sample, identify and interpret fungal exposure to these species, however no strategy serves all purposes as exposure is a complex and dynamic process confounded by spatial, temporal and geographic variations in airborne counts, in addition to the inadequacies of the immunodiagnostic techniques available. To date, the interpretation of personal exposure and sensitisation to fungal allergens has been restricted to a few select species and the contribution of other genera, airborne hyphae and fragmented conidia to allergic disease are all poorly understood. The aim of the thesis was to utilize the Halogen Immunoassay (HIA) to diagnose fungal allergic sensitisation, to investigate the distribution and factors influencing allergens of fungi in the air and to understand what is actually inhaled in exposure settings. The novelty of the HIA derives from its unique ability to provide allergen sources that are actively secreted by the collected fungal spores and hyphae, which are bound to protein binding membranes (PBM) and then immunoprobed.

In Chapter 2, the HIA was compared to the commercial *in vitro* Pharmacia UniCap assay (CAP) and the *in vivo* skin prick test (SPT), using 30 sera from subjects SPT positive to *Aspergillus fumigatus* and/or *Alternaria alternata* and 30 who were SPT negative to these fungi but sensitised to non-fungal allergens. Sera were analysed by CAP and the HIA against *A. alternata*, *A. fumigatus*, *Cladosporium herbarum* and *Epicoccum purpurascens* and compared statistically. Between 3% and 7% of SPT negative sera were identified to have specific IgE towards *A. fumigatus* and *A.*

alternata, respectively. For the SPT positive sera, significant associations were found between the HIA and CAP scores for all fungal species tested ($P<0.0001$). Correlations between the HIA and SPT however, were weakly correlated for *A. alternata* ($r_s = 0.44$, $P<0.05$) but not for *A. fumigatus*.

In Chapter 3, personal exposure to indoor fungal aerosols was examined using the HIA to identify the fungal components that people were allergic to. Personal air sampling pumps (PASs) collected airborne fungal propagules onto PBMs for 2.5 hours indoors (n=21). Collected fungi were incubated overnight in a humid chamber to promote the germination of conidia. The membranes were then immunostained with pooled human *Alternaria* species-positive sera. All air samples contained fungal hyphae that expressed soluble allergens and were significantly higher in concentration than counts of conidia of individual well-characterised allergenic genera. Approximately 25% of all hyphae expressed detectable allergen compared to non-stained hyphae ($P<0.05$) and the resultant localisation of immunostaining was heterogeneous among hyphae. Fungal conidia of ten genera that were previously uncharacterised as allergen sources accounted for 8% of the total conidia that demonstrated IgE binding.

In Chapter 4, the number and identity of fungi inhaled by 34 adults in an outdoor community setting was measured over 2 hour periods by people wearing Intra-nasal air samplers (INASs) and compared to fungal counts made with a Burkard spore trap and filter air samplers worn on the lapel. Using INAS, the most prevalent fungi inhaled belonged to soil borne spores of *Alternaria*, *Arthrinium*, *Bipolaris*, *Cladosporium*, *Curvularia*, *Epicoccum*, *Exserohilum*, *Fusarium, Pithomyces*, *Spegazzinia, Tetraploa* and Xylariaceae species, in addition to hyphal fragments. These results showed that inhaled exposure in most people varied in a 2-fold range with 10-fold outliers. In addition, the INAS and personal air filters agreed more with each other than with Burkard spore trap counts. The analysis was further confounded by different sampling efficiencies, locations of devices and ability to visualise and count fungal propagules.

In Chapter 5, a double immunostaining technique based on the HIA was developed and applied to the conidia, hyphae and fungal fragments of *A. alternata*, *A. fumigatus* and *Penicillium chrysogenum* to discriminate between sources of allergens,

using IgE and to identify the fungi, using a fungal-specific antibody. The localisation of immunostaining was heterogeneous between both conidia and the state of germination with greater concentrations of double immunostaining detected following germination for each fungal species (P<0.0001). Fragmented *A. alternata* hyphae and morphologically indiscernible fragments could be identified for the first time using this technique.

In Chapter 6, the factors affecting the release of allergen from the spores of eleven different species were studied. For nine of eleven species, between 5.7% and 92% of spores released allergen before germination. Ungerminated spores of *P. chrysogenum* and *Trichoderma viride* did not release detectable allergen. After germination, all spores that germinated eluted allergen from their hyphae. Upon germination there was a significant increase in the percentage of spores eluting detectable allergen (P<0.0001) and the localisation of allergen along the hyphae varied between species. Increased elution of allergen post germination might be a common feature of many species of allergenic fungi following inhalation. Additionally, Chapter 6 explored the extent to which inhaled spores or hyphae germinate after deposition in the nasal cavity and thus cause exposure to allergens. Twenty subjects had their noses lavaged at three separate intervals, (1) at the beginning of the experiment, (2) after one hour indoors and (3) after one hour outdoors. The recovery of spores and hyphal fragments from the nasal cavity varied between individuals and was significantly greater after outdoor exposures. Germinated fungal spores were recovered often in high concentrations for *Aspergillus-Penicillium* species, however the proportion between ungerminated and germinated spores were much lower for other genera recovered.

Conclusions: Our analysis of cultured and wild-type fungi 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. Exposure is heterogeneous between individuals in the same geographic locality and the spectrum of fungal genera inhaled differs with the method of analysis. Many of the spores inhaled are likely to be allergenic, however upon germination there is an increased elution of allergen and this might be a common

feature of many fungal species following inhalation. This project also provides novel techniques to diagnose fungal allergy by immunostaining wild-type fungi to which a patient is exposed with the patient's own serum. Such an immunoassay combines environmental with serological monitoring on a patient specific basis and potentially avoids many problems associated with extract variability, based on the performance of current diagnostic techniques for fungal allergy.

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I would like to dedicate this thesis for what it is worth, firstly to Mum. I know you wanted me to pursue my love of science and find my place in life, however there were a number of sacrifices that were required. I regret not being by your side when you needed me most when you found out that you had secondary cancer and during your first couple of doses of chemotherapy. You gave up so much for me throughout your life and I hope I was able to make you happy when it was really hard for you. I just wish you could be here so I could give you a big hug. I love and miss you so much and you will always be with me. I would also like to thank my Dad as well as the rest of my family in the United States for their continued support both financially and keeping me sane through the bad times and for being there whenever I needed a chat. I love you all very much.

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Publications – Peer reviewed

1. **Green BJ**, Mitakakis TZ and Tovey ER. (2003) **Allergen detection from 11 fungal species before and after germination**. *Journal of Allergy and Clinical Immunology*, 111 (2): 285-289.

2. **Green BJ**, Sercombe JK, Tovey ER. (2005) **Fungal fragments and undocumented conidia function as new aeroallergen sources**. *Journal of Allergy and Clinical Immunology,* 115 (5): 1043-1048.

3. Tovey ER, **Green BJ**. (2005) **Measuring environmental fungal exposure**. *Medical Mycology,* 43 (suppl 1): 67-70.

4. **Green BJ**, Schmechel D, Tovey ER. **Detection of *Alternaria alternata* conidia and hyphae using a novel double immunostaining technique**. *Clinical and Diagnostic Laboratory Immunology*, 12 (9): 1114-1116.

5. **Green BJ**, Schmechel D, Sercombe JK, Tovey ER. **Enumeration and detection of aerosolized *Aspergillus fumigatus* and *Penicillium chrysogenum* conidia and hyphae using a novel double immunostaining technique**. *Journal of Immunological Methods* (In press – printing date, September 2005).

6. **Green BJ**, Yli-Panula, E and Tovey ER. **A new method (Halogen Immunoassay) for the detection of fungal allergens and the diagnosis of fungal allergy; a comparative study with conventional techniques**. *Allergology International* (In press – printing date, November 2005).

7. Sercombe JK, **Green BJ** and Tovey ER. **Recovery of germinating fungal conidia from the nasal cavity following environmental exposure.** *American Journal of Rhinology* (submitted – in review, June 2005).

8. **Green BJ**, O'Meara, T, Sercombe, J, and Tovey ER. **Interpretation of personal exposure to outdoor aeromycota in northern New South Wales, Australia.** *Annals of Agricultural and Environmental Medicine* (submitted – in review, July 2005).

Conference Abstracts

1. **Green BJ**, Mitakakis TZ and Tovey ER. (2002) **Allergen detection of nine different genera of the Fungi Imperfecti, pre- and post- germination.** AAAAI, New York. *Journal of Allergy and Clinical Immunology,* 109 (1): s138 – s139.

2. Hodgson JL, Kent RF, Hodgson DR, McAleese S, McGorum BC, **Green BJ** and O'Meara T. (2003) **Use of a Halogen assay for detection of IgE to specific aeroallergens in horses.** 21st Symposium of the Veterinary and Comparative Respiratory Society, San Antonio, Texas, October 2- 5, 2003.

3. **Green BJ**, Yli-Panula E, Sercombe JK, Tovey ER. (2003) **A new *in vitro* immunodiagnostic method for the detection of fungal allergy; comparison with the UniCap™ assay and skin prick tests.** Abstract, CRC for Asthma conference, Wollongong, Australia, November 24-25, 2003.

4. **Green BJ**, Yli-Panula E, Sercombe JK, Tovey ER. (2004) **Diagnosis of fungal allergy: Comparison of detection of allergens from germinating fungi (Halogen Method) with Pharmacia UniCap Assay and Skin Prick Tests.** Abstract, AAAAI, San Francisco. *Journal of Allergy and Clinical Immunology*, 113 (2): s288 – s289.

5. Sercombe JK, **Green BJ** and Tovey ER. (2004) **Recovery and identification of fungal spores from the nasal cavity.** Abstract, AAAAI, San Francisco. *Journal of Allergy and Clinical Immunology,* 113 (2): s231.

6. O'Meara T, **Green BJ**, Sercombe JK and Tovey ER. (2004) **Interpretation of pollen exposure data.** Abstract, AAAAI, San Francisco. *Journal of Allergy and Clinical Immunology,* 113 (2): s62 – s63.

7. Tovey ER, **Green BJ**. **Measuring environmental fungal exposure.** Advances Against Aspergillosis, San Francisco, California, September 9-11, 2004.

8. **Green BJ**, Sercombe JK, Schmechel D, Tovey ER. **Detection of allergenic conidia and hyphae of *Alternaria alternata* and *Aspergillus fumigatus* using a novel double immunostaining technique.** AAAAI, San Antonio, Texas, March 18-22, 2005.

9. Sercombe JK, **Green BJ**, Tovey ER. **Novel technique allows simultaneous recognition of many genera of environmental fungi and conidia as allergen sources.** AAAAI, San Antonio, Texas, March 18-22, 2005.

Letters and Editorials

1. Leung DYM, Nelson HS, Szefler SJ, Busse WW. **The Editors' Choice; New fungal aeroallergen sources: Airborne hyphae, fragments, and undocumented conidia.** *Journal of Allergy & Clinical Immunology,* 115 (5): 895-896.

International Presentations

1. **Measuring environmental fungal exposure.** Advances Against Aspergillosis, San Francisco, California, September 9-11, 2004.

2. **Enumeration of inhalable fungi and new advances in the diagnosis of fungal allergy.** National Institute of Occupational Safety and Health, Centers for Disease Control and Prevention (CDC). Morgantown, West Virginia, November 29, 2004.

Published article abstracts

1. Green BJ, Mitakakis TZ, Tovey ER. Allergen detection from 11 fungal species before and after germination. *Journal of Allergy & Clinical Immunology*. 2003; 111: 285-289.

Abstract

Background: Allergens dispersed by airborne fungal spores play an important, but poorly understood role in the aetiology and exacerbation of asthma. Previous studies suggest that after germination, spores of *Alternaria* and *Aspergillus* release greater quantities of allergen than before germination. It is unknown if this is true of other allergenic fungi.

Objective: Our purpose was to investigate the release of allergen from a range of individual fungal spores before and after germination.

Methods: Allergen expression from spores of *Alternaria alternata*, *Cladosporium herbarum*, *Aspergillus fumigatus*, *Botrytis cinerea*, *Epicoccum nigrum*, *Exserohilum rostratum*, *Penicillium chrysogenum*, *Stemphylium botryosum, Curvularia lunata, Trichoderma viride* and *Bipolaris spicifera* was examined by Halogen immunoassays, using pooled serum IgE from fungal-allergic subjects. Spores were deposited onto protein binding membranes direct from culture. To germinate spores, samples were incubated in high humidity at room temperature for 48 hours. Ungerminated and germinated samples were then laminated with an adhesive film and immunostained by the Halogen assay. The samples were examined by light microscopy and positive counts (haloed particles) were expressed as percentages of total spores.

Results: For 9 of 11 species, between 5.7 - 92% of spores released allergen before germination. Spores of *Penicillium* and *Trichoderma* did not release detectable allergen.

After germination, all spores that germinated exhibited allergen elution from their hyphae. 8 of 11 species showed a significant increase ($p<0.05$) in the percentage of spores eluting detectable allergen. Localization of allergen along the hyphae varied with species, such that some eluted allergen mainly from hyphal tips and septal junctions, whereas others eluted allergen along the entire length.

Conclusions: Increased elution of allergen following germination may be a common feature of many species of allergenic fungi. Although allergens from both spores and hyphae were recognised by human IgE, the extent that human 'exposure' occurs to allergens eluted from inhaled spores or from hyphae that germinate following deposition in the respiratory tract, remains to be explored. The patterns of allergen expression may affect the clinical response to such exposure.

2. Green BJ, Sercombe JK, Tovey ER. Fungal fragments and undocumented conidia function as new aeroallergen sources. *Journal of Allergy & Clinical Immunology*. 2005; 115 (5): 1043-1048.

Abstract

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<0.05). Resultant immunostaining of fungal hyphae was heterogeneous, and approximately 25% of all hyphae expressed detectable allergen compared with nonstained hyphae (P<0.05). 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.

3. Tovey ER, **Green BJ**. Measuring environmental fungal exposure. *Medical Mycology*. 2005; 43 (suppl 1): 67-70.

Abstract

Airborne fungi are ubiquitous in the environment and human exposure is inevitable. Such fungi differ greatly in their taxonomic, physical, ecological, behavioural, and pathogenic characteristics. Many strategies have evolved to sample, identify and interpret fungal exposure and their choice is determined by the hypotheses involved. While fungi can be sampled directly from surfaces, results do not generally reflect human exposure. For this reason, airborne spores are commonly sampled, by either filtration or impaction, using volumetric air samplers. Identification is commonly performed by either culture on nutrient medium or light microscopy using morphological criteria, although new techniques using DNA probes or characteristic antigens or toxins continue to be developed. Interpretation of such exposure data is both complex and contentious, but while there are numerous recommendations there is no consensus on exposure thresholds. A better understanding of the complex pathogenic roles of fungi and susceptibilities of their hosts will enable refinement of techniques for sampling and interpretation.

4. **Green BJ**, Schmechel D, Tovey ER. Detection of *Alternaria alternata* conidia and hyphae using a novel double immunostaining technique. *Clinical and Diagnostic Laboratory Immunology*, 12 (9): 1114-1116.

Abstract

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

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List of Abbreviations

IgE	Immunoglobulin epsilon (ε)
TLRs	Toll-like receptors
IL	Interleukin
TNF	Tumour necrosis factor
IFN	Interferon
APCs	Antigen-presenting cells
MHC	Major histocompatibility complex II
kDa	Kilodaltons
SEM	Scanning electron microscopy
PCR	Polymerase chain reaction
CFUs	Colony forming units
AFS	Allergic fungal sinusitis
SPT	Skin prick test
INAS	Intra-nasal air samplers
HIA	Halogen immunoassay
CAP	Pharmacia UniCap
PBM	Protein binding membrane
SM	Skim milk
PBS	Phosphate buffered saline
PAS	Personal volumetric samplers
IOM	Institute of Occupational Medicine
MPBM	Mixed cellulose ester protein binding membrane
BSA	Bovine serum albumin
SaD	Sampling day
ANOVA	Analysis of variance
ICC	Intra class correlation co-efficient
mAb	Monoclonal antibody
HRP	Horseradish peroxidase
pAb	Polyclonal antibody
PAS	Periodic acid Schiff
V8	Vegetable juice nutrient agar
RB	Rose-Bengal chloramphenicol nutrient agar
CFS	Chronic fungal sinusitis