

Halogen Immunoassay, a New Method for the Detection of Sensitization to Fungal Allergens; Comparisons with Conventional Techniques

Brett James Green^{1,2}, Eija Yli-Panula^{1,3} and Euan Roger Tovey^{1,2}

ABSTRACT

Background: Accurate diagnosis of allergy to specific fungal species is confounded by the variability in allergens occurring with different diagnostic systems. We compared the halogen immunoassay (HIA), which uses allergens expressed by freshly germinated spores that are bound to protein binding membranes (PBM), with the commercial Pharmacia UniCap[®] assay (CAP) and with skin prick tests (SPT).

Methods: Serum from 60 subjects was used; 30 were SPT positive and sensitized to at least one of *Alternaria alternata* or *Aspergillus fumigatus* and the other 30 were SPT negative to these fungi but known to be sensitized to non-fungal allergens. All sera were analyzed by CAP against *A. alternata*, *A. fumigatus*, *Cladosporium herbarum* and *Epicoccum purpurascens*. For HIA, spores from reference cultures belonging to these four species were germinated on PBM, laminated and then probed with each serum. Two independent observers using an ordinal ranking system quantified the intensity and occurrence of the resultant immunoglobulin E (IgE) immunostained haloes around spores and this was statistically compared with the results of the two conventional immunodiagnostic techniques.

Results: Germinated conidia of each species expressed detectable allergen in the HIA. The agreement between the ordinal rank scores assigned by the pair of observers was very good ($k \geq 0.8$) and only differed for *A. fumigatus* ($k = 0.66$). Between 3% and 7% of SPT negative sera was identified by HIA to have specific IgE towards *A. fumigatus* and *A. alternata*. For all four species tested there were strong correlations between HIA and CAP ($P < 0.0001$). However the correlation of both HIA and CAP to SPT was weaker for *A. alternata* ($r_s = 0.44$, $P < 0.0153$) and absent for *A. fumigatus*.

Conclusions: Overall, the HIA is a new immunodiagnostic technique for the detection of sensitization to fungal allergens that correlates significantly with CAP and to a lesser extent with SPT. This may be due to extract variability and system differences. The significance of this derives from the unique ability of the HIA to measure IgE antibodies to the undegraded allergens that are actively secreted by germinating conidia and hyphae. These are the natural agents of exposure to fungi, and as such, are most likely to be relevant to clinical disease.

KEY WORDS

allergen, conidia, fungi, immunoassay, mold

INTRODUCTION

More than 80 species of fungi are suspected of inducing immunoglobulin E (IgE) mediated hypersensitivity.¹ The most commonly studied fungal species are

Alternaria alternata, *Aspergillus fumigatus*, *Cladosporium herbarum* and *Epicoccum purpurascens*, which are prevalent aeroallergen sources throughout the world and allergy to them is a risk factor for allergic rhinitis,² asthma,³ and even death.⁴ Exposure to mold

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occurs both indoors and outdoors and can vary between individuals, geographical location, time and air flow.⁵ The incidence of respiratory fungal allergic disease varies geographically, although it is estimated that 20 to 30% of atopic individuals and up to 6% of the general population are atopic to fungi.⁶

Fungal allergic disease can be diagnosed by *in vivo* skin prick test (SPT), *in vitro* detection of allergen specific serum IgE antibodies (RAST, Pharmacia CAP System™) and in some cases by provocative challenge tests.⁷ The most frequently used diagnostic test is the SPT, which uses a commercial fungal extract.^{8,9} It is well recognized that such extracts are heterogeneous in allergen content and vary between manufacturers, batches and strains. This has contributed to the slower rate of allergen standardization, compared with other aeroallergen sources.^{8,9} Furthermore, the lack of concordance between fungal *in vivo* and *in vitro* diagnostic tests has added to the clinical uncertainty about the accuracy and effectiveness of current fungal allergen diagnostic systems.¹⁰⁻¹³ Within the community, the median wheal size for fungal allergy lies close to the cut-off value conventionally used to discriminate between positive and negative responses.¹⁴ It is likely that the variability of existing extracts, the use of a few selected extracts to represent a broader range of fungi and the uncertainty of an appropriate cut-off for a positive response have all contributed to the under-diagnosis of the prevalence of fungal allergy.

Until now, the collection of wild-type fungi from a patient's environment and directly demonstrating allergic sensitization to the same fungal particles has been a technically formidable task.¹⁵ The recently developed halogen immunoassay (HIA) enables the simultaneous visualization of individual particles collected by volumetric air sampling, together with their expressed antigens following their immunostaining with human IgE.¹⁶ Originally developed as a novel analytical technique for a range of common aeroallergen sources including house dust mite,¹⁷ cat,¹⁸ cockroach¹⁹ and pollen,²⁰ the HIA has recently been successfully adapted to the detection of allergens expressed by germinated fungal conidia.^{21,22} These preliminary studies have demonstrated that the HIA provides a mechanism to test for reactivity to the allergens that are expressed by germinating fungi and thus simulates natural exposure to conidia.

In this study we compare two groups of sera from subjects classified as being either SPT positive or negative to two common fungi (*A. alternata* and *A. fumigatus*). The analysis of allergy to these species, in addition to two more (*C. herbarum* and *E. purpurascens*) by the HIA, using fungi derived from culture, were compared with the conventional diagnostic systems of SPT and the Pharmacia UniCap® assay (CAP).

METHODS

STUDY POPULATION

Atopic serum was sourced from a previous study of adult respiratory diseases in different Australian communities.²³ Ages of the subjects ranged from 17 to 50 years; however as samples were de-identified the calculation of a median age was not possible. The subjects were classified as having allergy-associated disease if they previously had a history of wheeze, asthma or hay fever.²⁴ The local research ethics committee approved the study protocol and the subjects gave written informed consent following a full explanation of the study.

SKIN TESTING PROCEDURES

SPT was performed with a panel of eleven common standardized aeroallergen and mold extracts using a standardized procedure and lancets from Hollister-Stier Laboratories (Hollister-Stier, Spokane, WA, USA).²⁵ The mean of the longest and perpendicular wheal size was recorded after 15 minutes and expressed as mean wheal mm. Sensitization to mold was defined as having a minimum wheal diameter of 3 mm or greater, 15 minutes after the testing procedure to one of either *A. alternata* or *A. fumigatus* SPT extracts.²⁶

MEASUREMENT OF TOTAL AND SPECIFIC IgE

Following the SPT, sera was obtained from consenting subjects for *in vitro* diagnostic testing and stored in aliquots at -70°C until required. Sera from 60 atopic subjects were selected to be tested; 30 were sensitized to at least one of *A. alternata* or *A. fumigatus* extracts and the other 30 subjects were SPT negative to these fungi but sensitized to other non-fungal allergens. Undiluted sera were serologically tested at the Clinical Immunology Laboratory (Central Sydney Laboratory Service, NSW, Australia) for specific IgE antibodies to *A. alternata* (m6), *A. fumigatus* (m3), *C. herbarum* (m2) and *E. purpurascens* (m14) using the CAP assay according to the manufacturers' instructions (Pharmacia CAP System™, Pharmacia and Upjohn Diagnostics AB, Uppsala, Sweden). The positive cut-off for the specific IgE assays was 0.35 kU_A/L and was calibrated against the World Health Organization standard 75/502 for IgE in the range 0.35–100 kU_A/L.

FUNGAL ISOLATES

Fungal isolates of *A. alternata* (28008), *A. fumigatus* (28004), *C. herbarum* (28006) and *E. purpurascens* (28007) were supplied by the Queensland Department of Primary Industries (Brisbane, Australia). Isolates of each species were subcultured from stock sources and grown for 10 days at 24.9°C on vegetable juice nutrient media until sufficient sporulation occurred.

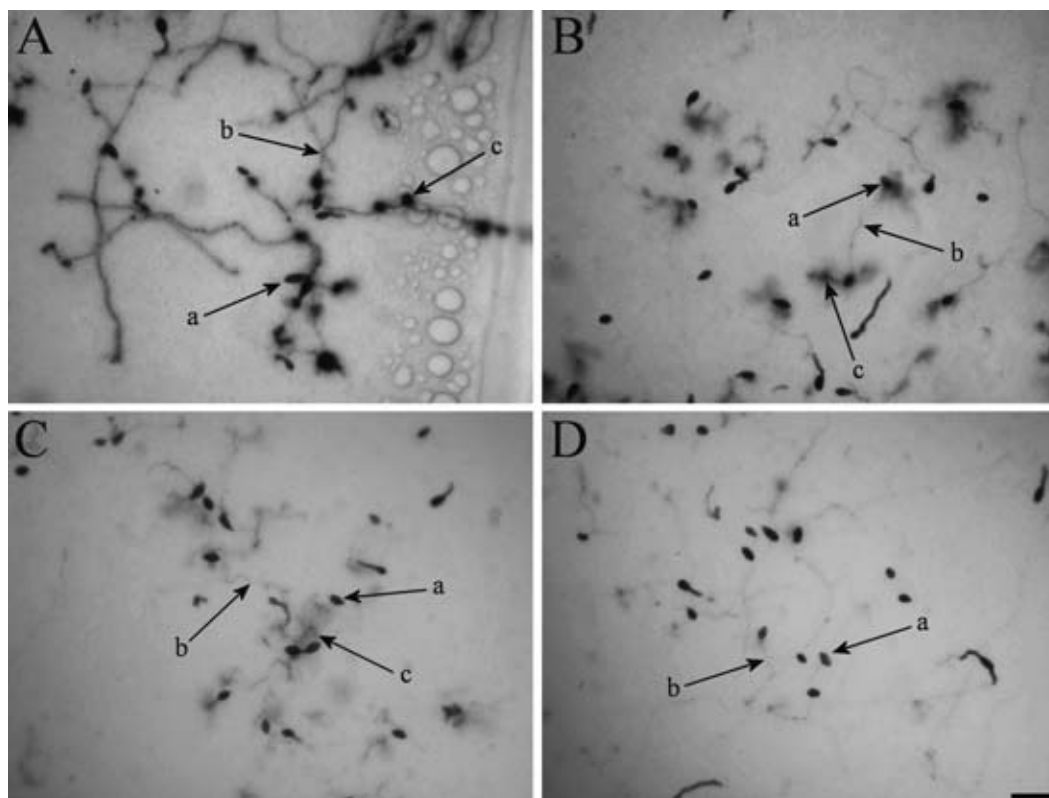


Fig. 1 Germinated *Alternaria alternata* showing different intensities of immunostaining of conidia (arrow a) and hyphae (arrow b) using the halogen immunoassay to detect the binding of human IgE (arrow c) to expressed allergens. Rank scoring is as follows; **A.** high positive (3 +). **B.** medium positive (2 +). **C.** low positive (1 +) and **D.** negative (0). Scale Bar — 50 μ m.

CULTURED SPORE SAMPLING

Conidia were aerosolized from culture plates using an air jet and then collected by suction onto a polyvinylidene difluoride protein binding membrane (PBM; 0.45 μ m pore size, 25 mm diameter) that had been pre-incubated with 30% sucrose and then dried as described previously.²¹ After collection, the membrane was kept in a humid box overnight at room temperature to allow the germination of conidia, which has been shown previously to significantly increase the expression of allergen from fungi.²¹ Germinated conidia samples were permanently laminated to the membrane by overlaying it with a clear water-based adhesive film (Woolcock Institute of Medical Research, Sydney, Australia).

HALOGEN IMMUNOSTAINING OF CULTURED SPORE SAMPLES

Briefly in the HIA, previously laminated PBMs with germinated conidia were immersed in 80% methanol until wet, rinsed in deionized water and incubated in borate buffer (pH 8.2) for 4 hours to enable allergens to elute and bind to the PBM in close proximity to the germinated conidia. Membranes were blocked in 5% skim milk (SM) in phosphate buffered saline (PBS,

pH 7.4) for 45 minutes and then incubated overnight at 4°C with individual patient sera (dilution 1 : 3, vol/vol-100 μ l patient sera) in 2% SM-PBS-0.05% Tween 20 (Tw). After the primary antibody incubation, the membranes were rinsed three times in PBS-Tw and incubated for 1.5 hours with biotinylated goat anti-human IgE (Kirkegaard&Perry Laboratories, Gaithersburg, MD, USA) diluted 1 : 500 in 2% SM-PBS-Tw. This was followed by an incubation for 1.5 hours with ExtrAvidin alkaline phosphatase conjugate (Sigma Chemical Co, St Louis, MO, USA), diluted 1 : 1000 in 2% SM-PBS-Tw. For immunostaining, all samples were incubated with the alkaline phosphatase substrate NBT/BCIP (Pierce Chemical Co, Rockford, IL, USA). Staining was then monitored for 20 minutes periodically until an optimum purple precipitate was achieved. Positively immunostained germinated conidia have visible immunostaining of the allergen that has been bound to the PBM in close proximity to the conidia or hyphae. Entire PBMs were examined at a magnification of 200x by standard light microscopy. Three replicates were evaluated and the experiment was repeated.

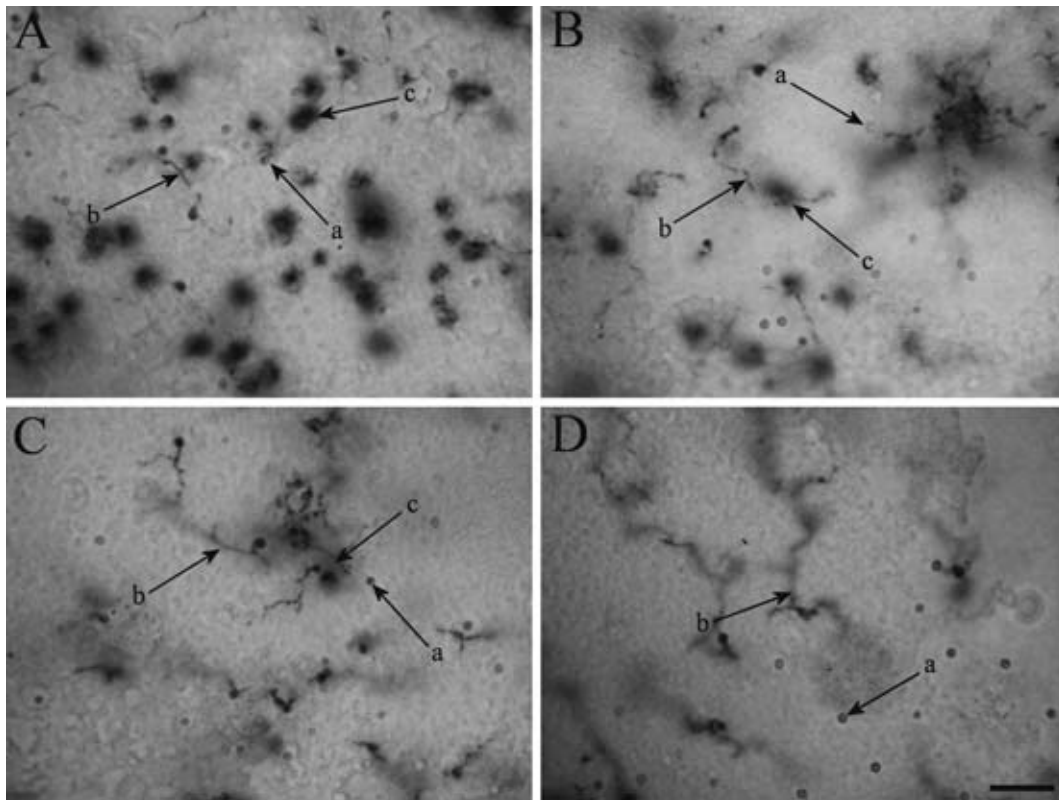


Fig. 2 Germinated *Aspergillus fumigatus* showing different intensities of immunostaining of conidia (arrow a) and hyphae (arrow b) using the halogen immunoassay to detect the binding of human IgE (arrow c) to expressed allergens. Rank scoring is as follows; **A.** high positive (3 +). **B.** medium positive (2 +). **C.** low positive (1 +) and **D.** negative (0). Scale Bar — 20 μ m.

HALOGEN IMMUNOASSAY RANK SCORE

Two independent observers using a subjective ordinal ranking system quantified the intensity, occurrence and localization of the resultant IgE immunostained haloes around germinated conidia. The intensity of the precipitate staining around hyphae and germinated conidia was estimated as 3+ for strong immunostaining (dark purple), 2+ for moderate immunostaining (medium purple), 1+ for weak immunostaining (light purple) and 0 for no immunostaining reaction. For each species, a series of photomicrographs showing different intensities of immunostaining were used as references for the classification of ordinal rank scores.

STATISTICAL ANALYSIS

The statistical agreement between the ordinal rank scores that were assigned by each independent observer was calculated using Cohen's kappa statistic²⁷ using Analyse-It software (Analyse-It for Microsoft Excel, Version 1.68, Analyse-It Software Ltd., Leeds, United Kingdom). In the Kappa test for agreement, the resultant co-efficient (k) gives values between -1 and 1. When there is exact agreement for the two measures of all subjects the kappa statistic equals 1.

A value less than 0.6 represents a non-significant agreement between two variables. The kappa statistic was interpreted as follows: 1.00–0.81 = very good agreement; 0.80–0.61 = good agreement; 0.60–0.41 = moderate agreement; 0.40–0.21 = poor agreement; and 0.20–0.00 = no agreement.

The associations between the HIA, CAP and SPT were analyzed for significance using the non-parametric Spearman correlation analysis (Analyse-It for Microsoft Excel, Version 1.68, Analyse-It Software Ltd., Leeds, United Kingdom). The criterion for significance for all analyses was $P < 0.05$. Except otherwise noted, data are expressed as Spearman coefficients (r_s) and P values.

RESULTS

LOCALIZATION OF IMMUNOSTAINING

The fungi showed differing patterns of allergen expression from both the hyphae and the site of conidial germination following HIA analysis, which is consistent with earlier observations.^{21,22} Resultant immunostaining for high positive *A. alternata* sera was heterogeneous and confined to hyphal septal junctions and at the tips of hyphae often in high concentrations (Fig. 1A). For medium and low positive ordi-

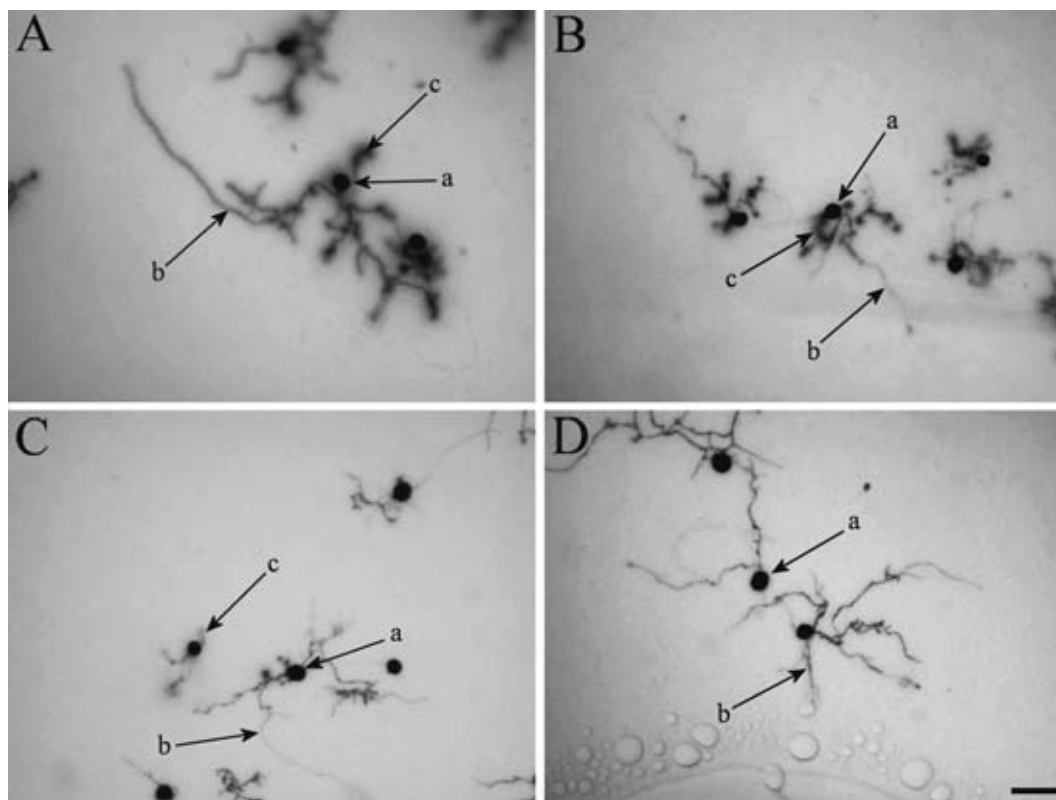


Fig. 3 Germinated *Epicoccum purpurascens* showing different intensities of immunostaining of conidia (arrow a) and hyphae (arrow b) using the halogen immunoassay to detect the binding of human IgE (arrow c) to expressed allergens. Rank scoring is as follows; **A.** high positive (3 +). **B.** medium positive (2 +). **C.** low positive (1 +) and **D.** negative (0). Scale Bar – 50 μ m.

nal rank scores, the intensity of resultant immunostaining qualitatively weakened and antigen expression was restricted to along the entire hyphae and the sites of conidial germination (Fig. 1B, 1C).

For germinated conidia belonging to *A. fumigatus* (Fig. 2), *E. purpurascens* (Fig. 3) and *C. herbarum* (data not shown) resultant immunostaining was homogeneous. The expression of allergen for each of these respective species was localized around the perimeter of the hyphae, especially at the site of conidial germination. The concentration, intensity and the localization of resultant immunostaining qualitatively decreased with each respective ordinal rank score. Although conidia and hyphae themselves showed some staining within the negative control serum, this was confined to the organelles themselves and there was no immunostaining of the PBM.

INTER-OBSERVER AGREEMENT

The intensity and occurrence of the resultant IgE immunostaining around germinated conidia and hyphae were quantified by two independent observers using a predefined ordinal rank scale. The agreement between the HIA rank scores assigned to the samples by each observer is demonstrated in Table 1. Overall,

the agreement between the observers for the ranking of *A. alternata*, *C. herbarum* and *E. purpurascens* was very good ($k \geq 0.8$). However, the agreement between *A. fumigatus* rank scores was slightly lower than that found for the other species and indicated good agreement ($k = 0.66$).

CORRELATIONS BETWEEN IMMUNODIAGNOSTIC TECHNIQUES

The results in Table 2 demonstrate the proportion of sera samples that were identified in each of the immunodiagnostic techniques to be either fungal positive or negative. For fungal SPT negative sera, 7% ($n = 2$) of serum samples were identified to be positive to *A. alternata* in the HIA, whereas 10% ($n = 3$) were identified in CAP. Similarly, 3% ($n = 1$) and 7% ($n = 2$) of the fungal negative sera were identified to be positive to *A. fumigatus* in the HIA and CAP, respectively, whereas only one negative serum sample was established by CAP alone to be positive to *E. purpurascens* (Table 2). For the fungal positive SPT sera samples, the resultant proportion of positive and negative sera within this group was heterogeneous for each of the species (Table 2). There were greater numbers of sera identified to be positive in the HIA to *A. alter-*

Table 1 Inter-observer agreement between the HIA ordinal rank scores that were assigned by each independent observer.

Fungal species	HIA ordinal rank score [†]								Agreement [‡]
	3+		2+		1+		0		
	T1	T2	T1	T2	T1	T2	T1	T2	
<i>Alternaria alternata</i>	2 (3)	3 (5)	6 (10)	6 (10)	20 (33)	20 (33)	32 (53)	31 (52)	0.81
<i>Aspergillus fumigatus</i>	2 (3)	2 (3)	3 (5)	3 (5)	11 (18)	7 (12)	44 (73)	48 (80)	0.66
<i>Cladosporium herbarum</i>	1 (2)	1 (2)	2 (3)	1 (2)	19 (32)	15 (25)	38 (63)	43 (72)	0.79
<i>Epicoccum purpurascens</i>	2 (3)	2 (3)	4 (7)	3 (5)	19 (32)	21 (35)	35 (58)	34 (57)	0.82

[†] Values presented represent the total number of serum samples assigned to each HIA ordinal rank score by the observer (T1 or T2), with the percentage frequency given in parentheses.

[‡] Agreement identifies the inter-observer agreement between HIA ordinal rank scores as indicated by the kappa statistic (*k*) for each fungal species.

Table 2 The number of serum samples identified by each immunodiagnostic technique to be either sensitive to fungi (+ ve) or not sensitive to fungi (– ve).

Fungal species	Immunodiagnostic technique [†]					
	HIA		CAP		SPT	
	+ ve	– ve	+ ve	– ve	+ ve	– ve
Fungal – ve sera (<i>n</i> = 30)						
<i>Alternaria alternata</i>	2 (7)	28 (93)	3 (10)	27 (90)	0 (0)	30 (100)
<i>Aspergillus fumigatus</i>	1 (3)	29 (97)	2 (7)	28 (93)	0 (0)	30 (100)
<i>Epicoccum purpurascens</i>	0 (0)	30 (100)	1 (3)	29 (97)	ND [‡]	ND
<i>Cladosporium herbarum</i>	0 (0)	30 (100)	0 (0)	30 (100)	ND	ND
Fungal + ve sera (<i>n</i> = 30)						
<i>Alternaria alternata</i>	24 (80)	6 (20)	20 (66)	10 (33)	19 (63)	11 (37)
<i>Aspergillus fumigatus</i>	9 (30)	21 (70)	12 (40)	18 (60)	18 (60)	12 (40)
<i>Epicoccum purpurascens</i>	23 (77)	7 (23)	15 (50)	15 (50)	ND	ND
<i>Cladosporium herbarum</i>	17 (57)	13 (43)	14 (47)	16 (53)	ND	ND

[†] Values presented represent the total number of serum samples identified by each immunodiagnostic technique to be either fungal positive or negative with the percentage frequency given in parentheses.

[‡] ND = not detected

nata, *E. purpurascens* and *C. herbarum* than in CAP and SPT (Table 2). Furthermore, only 30% and 40% of fungal positive sera was established to be positive to *A. fumigatus* in the HIA and CAP, respectively, compared with SPT in which 60% of the sera was positive to *A. fumigatus*.

The correlations between the HIA, SPT and CAP are shown in Table 3. Significant correlations were found between the HIA and CAP for each of the four fungal species studied, in particular *A. alternata* ($r_s = 0.79$, $P < 0.0001$), *A. fumigatus* ($r_s = 0.67$, $P < 0.0001$) and *C. herbarum* ($r_s = 0.66$, $P < 0.0001$). However for *E. purpurascens*, the association between HIA and CAP was weaker ($r_s = 0.52$, $P < 0.0035$) than for the other species (Table 3).

The associations between the HIA and SPT for *A. alternata* and *A. fumigatus* were less strong and consistent compared with the CAP correlations. For *A. alternata* there was a significant but weak correlation between the two techniques ($r_s = 0.44$, $P < 0.0153$), while there was no correlation between HIA and SPT

for *A. fumigatus* (Table 3). Similarly, there was a significant but weak correlation between CAP and SPT for *A. alternata* ($r_s = 0.43$, $P < 0.0182$) and again no correlation for *A. fumigatus* (Table 3).

DISCUSSION

This study showed that as an *in vitro* diagnostic technique to detect fungal-specific IgE in human serum, the HIA correlated well with the conventional allergy diagnostic systems of CAP and to a lesser extent with SPT. This finding represents a new approach to the detection of sensitization to fungal allergens, which may have significant clinical advantages as allergens detected by HIA are analogous to those released during natural exposure. This potentially avoids many of the confounding factors that limit both SPT and the *in vitro* tests, which depend on retaining the stability of allergens over prolonged periods of storage. The novelty of the technique can be additionally applied to fungal species for which conventional reagents are not available.

Table 3 Spearman correlation results for the SPT fungal positive sera between the three-immunodiagnostic techniques.

Fungal species	Immunodiagnostic technique [†]		
	HIA Rank Score	SPT	CAP
<i>Alternaria alternata</i>			
HIA Rank Score	1	0.44 (0.0153)[‡]	0.79 (0.0001)
SPT (mm)		1	0.43 (0.0182)
CAP (kU _A /L)			1
<i>Aspergillus fumigatus</i>			
HIA Rank Score	1	− 0.04 (0.8095)	0.67 (0.0001)
SPT (mm)		1	− 0.02 (0.9249)
CAP (kU _A /L)			1
<i>Cladosporium herbarum</i>			
HIA Rank Score	1	—	0.66 (0.0001)
SPT (mm)		—	—
CAP (kU _A /L)			1
<i>Epicoccum purpurascens</i>			
HIA Rank Score	1	—	0.52 (0.0035)
SPT (mm)		—	—
CAP (kU _A /L)			1

[†] Data show the non-parametric Spearman rs co-efficient and resultant *P* value.

[‡] Bold type identifies the Spearman correlation coefficients which were significant (*P* < 0.05).

Sera previously categorized as either SPT negative or positive to at least one of two fungal extracts were found to show only a moderate correlation on retesting by HIA and CAP to those two fungi, while the HIA and CAP showed good overall correlation with each other as *in vitro* assays using four fungal species. For the HIA and SPT, the high concordance for *Alternaria* and low concordance for *Aspergillus* is likely to, in part, be a function of the size and antigen production by the germinated conidia of these different genera. *Alternaria* spores are large (~25 µm diameter) and produce copious amounts of allergen, which is readily visualized in the assay. In contrast, *Aspergillus* spores have smaller dimensions (~3 µm diameter) and consequently produce minimal amounts of allergen that in some cases may have been below the detection thresholds of the assay. Overall, however, the HIA was the most sensitive, and in the comparison of four fungal species by HIA and CAP, 73 tests were positive by HIA compared with 61 by CAP and 3 subjects classified as SPT negative were positive by HIA. In other studies, comparing CAP/RAST and SPT, the concordance for fungi is low, varying from 39 to 57%,¹⁰⁻¹² while for common allergens such as mites, pollens and animals it is usually around 90%.²⁸

Measurement of the subject's allergic response to an allergen, whether in terms of the quantity of specific IgE or SPT wheal diameter, involves introducing categories into what occurs naturally as a continuous variable. Defining a boundary for a clinically relevant skin test or *in vitro* response is arbitrary and is only loosely linked to the probability of any clinical out-

come. Conventionally with skin tests, a wheal size of ≥3 mm is often taken as this cut-off point,²⁹ although rarely ≥2 mm or even ≥1 mm are used.³⁰ In a large population study, close to 50% of fungal allergic subjects had skin tests close to the 3 mm cut-off point,¹⁴ whereas for mite allergens, larger wheals were proportionately more common. In this study, we introduced three categories of classification for the HIA that were based on an observed intensity of immunostaining. All three categories showed some IgE binding of extracted allergen on the PBM and could be presumed to reflect a corresponding immune recognition had the spores been inhaled. The lower limit of detection in the HIA is also subject to variability, however this is not different than interpreting CAP or SPT scores, where lower levels of response (e.g. 2 mm SPT) are thought to be artifacts of a non-specific nature or clinically less relevant responses. While more objective measures of IgE binding in the HIA could presumably be made using a measurable label, such as fluorescent anti-IgE, this is technically complex in terms of the required image software and processing such improvements may not be required as visual scoring alone may be adequate for most purposes, as it is in the case of quantifying SPT results.

As a diagnostic system, SPTs are popular as they are less expensive, more convenient and are generally regarded as being more sensitive. However, they are also influenced by a range of biological and reading variables that confound the data.³¹ These include an individual's tissue histamine responsiveness, diurnal variation, site along the arm, operator variation, reading error and non-allergic contaminants in the

extracts. At least hypothetically, SPTs provide a more indicative biological response to an allergen. There are limited data comparing SPT with CAP using single purified allergens. These show that IgE binding by an allergen is only weakly associated with its ability to generate skin test wheals,³² and some allergens, for unclear reasons, seem to be biologically more potent than others.³³ Particularly with SPT extracts, there is probably ongoing degradation of some allergenic components during storage. In conventional *in vitro* assays, such as CAP, the allergens are more stable as they are bound to the solid phase through functional protein groups. However, fungal extracts contain a larger proportion of carbohydrate-associated allergens³⁴ than other common allergens, and as such, carbohydrate or glycoprotein allergens are less likely to be covalently coupled to the solid-phase used in CAP assays³⁵ than to the membranes used in the HIA, which have been shown to bind carbohydrate allergens such as mannan from *Candida albicans*.³⁶

Currently, to demonstrate allergy to individual fungal bioaerosols within an environment to which a subject is exposed, would require separate collections, culture and preparation of extracts for each species present. The HIA can provide the diagnosis of IgE mediated hypersensitivity to not only wild-type fungal conidia, but also airborne fungal hyphae, fragmented conidia and fungal genera not previously considered as allergen sources.^{37,38} The advantage of the HIA assay is that fungal species from a wide range of sources can be used. While in this study spores from culture were used, the technique lends itself to the use of wild-type fungi that the subject is exposed to. In addition, the conidia in the HIA are maintained for a short period at high humidity and naturally express their allergens, whereas those used for commercial extracts are cultured on artificial media, which could influence the expression of allergens. The significance of this technique derives from its unique ability to provide allergen sources that are actively secreted by the germinating spores and hyphae. This may reflect the allergens to which exposure occurs naturally after conidia are deposited on the mucous membrane. Thus the assay uses the un-degraded allergens that are the natural agent of exposure to fungi, and as such are most likely to be relevant to clinical disease.

Several experimental studies have shown that the germination of fungal conidia is an important process in the release of fungal allergens. The detection of Asp f 1 and Alt a 1, in addition to the immunostaining of IgE of a number of other fungal species significantly increases upon conidial germination in both culture^{21,22} and house dust.³⁹ Germinated conidia consist of growing hyphae, in which the hyphal tips are metabolically active and functionally differentiating regions that have been shown in cross sectional

studies to bind considerably greater amounts of Asp f 1 and human IgE than conidia.⁴⁰ Other fungal extract studies have also demonstrated that mycelium extracts bind significantly higher concentrations of IgE than spore extracts.⁴¹ In the present study, germination increased the detectable thresholds of the HIA and confirms earlier studies that ungerminated *Aspergillus* conidia cannot be accurately enumerated using the HIA.

In conclusion, the results of this study demonstrate that the use of freshly germinated spores in the HIA correlated well with conventional *in vitro* techniques and less well with SPT. The results also indicated a good agreement between the independent observer ordinal rank scores for each fungal species, which enabled the semi-quantification of the HIA. These outcomes form a background against which to determine the role of fungal exposure and fungal allergens in asthma, with the eventual aim of reducing its burden through better diagnosis, management and prevention.

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