

Optimized preparation of *Aspergillus fumigatus* extracts for allergy diagnosis

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Summary

Extracts of *Aspergillus fumigatus* are required for the measurement of specific antibodies that are important indices in the diagnosis of allergic bronchopulmonary aspergillosis (ABPA). This study investigated the effect of different culture conditions on the production and release of antigenic and allergenic proteins of *A. fumigatus*. Increasing the incubation temperature from 25°C to 37°C altered the production of proteins by the mycelium which resulted in the release of a greater number of proteins that reacted with precipitating antibodies. Static sporulating cultures produced a much wider antigenic spectrum than shake cultures although the number of precipitating proteins (5 and 3 respectively) and major IgE binding proteins (5 and 3 respectively) was not greatly altered. The widest range of proteins bound by precipitating antibody or IgE from ABPA serum were released into the culture filtrate during 28 day static incubation at 37°C. The resultant extract proved useful for screening patients for specific IgE and will provide a starting material for the identification of individual antigens or allergens.

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Introduction

Allergic bronchopulmonary aspergillosis (ABPA) is a complication of allergic reactions to pulmonary colonization by *Aspergillus fumigatus*. Increased levels of precipitating (IgG) antibodies and IgE specific for *A. fumigatus* are important criteria, in diagnosis of ABPA [1]. However extracts of *A. fumigatus* are difficult to standardize and differences in extract potency can lead to inconsistency in results of serological tests [2,3]. This leads to suggestions that a battery of extracts should be used [3]. Extracts of *A. fumigatus* are composed of different amounts of a range of protein components each with varying allergenic/antigenic activity. Variations in the number of antigenic components have been observed with strain [4] and to a lesser extent with different batches [5], although batch variations have been shown to be mainly quantitative [6]. The greatest variations have been noted with the phase of growth for antigen [7,8], and allergen [9] release. Extracts are made from mycelial or culture filtrate sources as conidial extracts exhibit little antigenicity [10,11].

The aim of this study was to examine the effects of

different growth conditions on antigenicity of extracts of *A. fumigatus* cultures in order to establish a method that leads consistently to the accumulation of a wide range of allergenic and antigenic components, and that would be suitable for screening patients for specific IgG and IgE antibodies. The specific antigens examined were Antigen 7 (Ag7), a major IgG binding component in ABPA [12], Antigen 13 (Ag13) (C antigen) an antigen important in the diagnosis of ABPA [13], and the minor antigen/allergen Antigen 5 (Ag5), thought to be important in initiating sensitization [11]. Ag5 is located on the surface of germinating conidia (the airborne spores of *A. fumigatus*), and is the primary antigenic component in the immune response in rabbits immunized with germinating conidia [11]. Antigen 7 has been found to have allergenic activity in 8/10 ABPA patients [14].

Methods

Antiserum

Pooled reference antiserum. Two Dutch Lop Rabbits were immunized with *A. fumigatus* antigen (5 mg/ml), in Freund's incomplete adjuvant with saline (1:2) alternating with bleeds every 2 weeks.

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Monospecific antisera. Antisera to Ag7, Ag13 and Ag5 were provided by Dr J. L. Longbottom and were prepared as described previously [12, 13 and 11 respectively].

Patient serum

Serum samples were obtained from patients attending the paediatric cystic fibrosis (CF) clinic at the Royal Brompton National Heart and Lung Hospital for annual assessment. A pool was made of serum from 14 patients with active or recent ABPA, who exhibited six of the seven features of ABPA described by Rosenberg *et al.* [1] (which include episodic bronchial obstruction, peripheral blood eosinophilia, skin-prick sensitivity, precipitating antibodies against *A. fumigatus*, elevated serum IgE, history of pulmonary infiltrates) and required steroid treatment. Serum samples from 25 ABPA patients and 108 non-ABPA patients were used to assess the value of the antigen extract in a radio-allergosorbent assay.

A. fumigatus isolates

A. fumigatus was isolated by plating out the sputum expectorated by three children (with cystic fibrosis and ABPA) onto malt agar (Oxoid) containing chloramphenicol (5 mg/ml) and incubating at 37°C. Fungal colonies were subcultured onto malt and Czapeck dox agar (Oxoid) and *A. fumigatus* identified according to the description by Thom and Raper [15]. The cultures were maintained on malt agar (Oxoid) incorporating chloramphenicol (5 mg/ml). Plug inocula (5 mm diameter) were cut from 4 day old cultures on semi-synthetic asparagine agar (SEA) (L-asparagine 0.7%, NH₄Cl 0.7%, K₂HPO₄ 0.13%, MgSO₄ 0.15%, sodium citrate 0.09%, ferric citrate 0.03%, glucose 1%, glycerol 2.5%), and immersed in sterile water with Tween 20 (0.05%) as a wetting agent. Conidial inocula were prepared by washing 4 day cultures on SEA plates with saline (5 ml), centrifuging and resuspended in saline (2500 conidia/ml).

Culture

The effect of temperature. Each of the three isolates were studied in duplicate. Sterile universal tubes (sterilin) containing synthetic asparagine medium (20 ml) were inoculated with mycelial plugs and rotated at 75 rev/min at 25°C or 37°C. At regular intervals cultures were removed in duplicate, filtered (Millipore, pore size 0.4 µm) and the wet mycelial weight and pH of the culture filtrate were measured.

The effect of culture type. Pyrex flasks (400 ml) containing synthetic asparagine medium (250 ml) were inoculated

with spore suspension (0.1 ml), and incubated at 37°C. Flasks were either rotated at 60 rev/min (agitated culture), or left to stand (static culture), at 37°C. At regular intervals flasks were removed and treated as above.

Antigenic preparations

The pH of the culture filtrates were measured before they were dialysed overnight against distilled water, freeze dried, and subsequently reconstituted in equal volumes of saline (100 µl). Mycelia were disrupted using a blender (Braun Multiprac, Middlesex, U.K.), sonicated (MSE Soniprep 150, Fisons Scientific Equipment, Loughborough, U.K.) and extracted overnight in phosphate buffered saline (PBS) with phenol (1%) solution at 4°C before being filtered (0.5 µm) to remove insoluble material and treated as above.

Immunochemical techniques

Crossed immunoelectrophoresis (CIE). This was used to visualize the total antigenicity of the extracts with human and hyperimmune rabbit sera. The method was carried out as described previously [6]. Briefly, glass plates (8 × 8 cm) were coated with agar (1%) (electran, BDH) in barbitone buffer (1:1 dilution of stock: sodium barbitone, 20.6 g, barbitone, 3.7 g, distilled water 1 l; pH 8.65). The extract (3 µl) was electrophoresed (20 mA, 90 min) in the first dimension and in the second dimension (10 mA, 18 hr) against hyperimmune rabbit antisera (11.5 µl/cm²) in the main gel. Proteins bound by patient precipitating antibody were identified by using an intermediate gel incorporating pooled ABPA serum (90 µl/cm²) or no serum as control. The precipitin peaks were visualized by staining with Coomassie brilliant blue R250 (0.5%) (Sigma).

Crossed radio immunoelectrophoresis (CRIE). This was used to identify protein peaks binding patient IgE. The above plates were overlaid with serum diluted in PBS with BSA (3%) (1:2) overnight, washed, then incubated with anti-human IgE labelled with ¹²⁵I (Phadebas, Pharmacia, Sweden) washed (3 ×) and exposed to direct exposure X-ray film (Kodak X-Omat) for 7 days at room temperature.

Rocket immunoelectrophoresis. The extract (3 µl) were electrophoresed (10 mA, 18 hr) into agar incorporating monospecific antisera (20 µl/cm²), and treated as in CIE.

Radio allergosorbent assay. The freeze dried antigen extract (10 mg) (culture filtrate after 28 days static culture) was conjugated to CNBr activated sepharose (1 g) (Pharmacia) to form the antigen particle complex

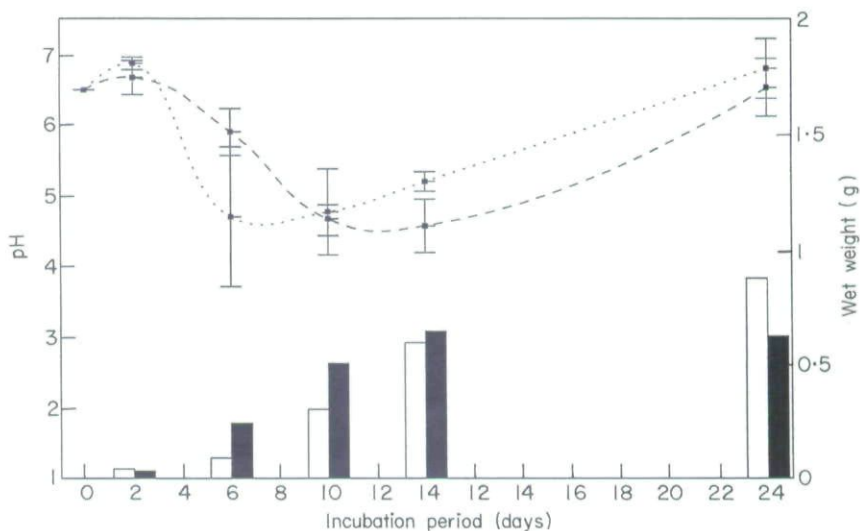


Fig. 1. Changes in culture filtrate pH at 25°C (---) and 37°C (···) (three isolates) and mycelial wet weight at 25°C (□) and 37°C (■) (isolate a) of *A. fumigatus* sampled in duplicate (bars indicate one standard deviation).

(APC). The APC (100 µl at 4%) was incubated (12 hr) with sera (50 µl), washed and incubated (12 hr) with anti-human IgE ¹²⁵I conjugate (50 µl) (Pharmacia Phadebas kit, Sweden), after washing. The % uptake of ¹²⁵I by the APC was calculated, and the results classed as follows, 0 (0–5%), 1 (6–15%), 2 (16–25%), 3 (26–35%), 4 (36–45). The commercial test used was *A. fumigatus* RAST discs (Pharmacia). A positive result defined as greater or equal to Class 2.

Statistical analysis

The Wilcoxon signed rank sum test was used to compare the results of each isolate (with duplicates) under the different conditions of growth.

Results

The effect of incubation temperature on growth and antigenicity

Growth. At both 25°C and 37°C, after an initial lag period when the pH rose slightly and little growth occurred, there was an increase in mycelial weight and a sharp drop in pH. The pH then rose as the mycelial mass continued to increase before reaching a stationary phase. The initial growth rate (day 6–10) was higher at 37°C than at 25°C. However, the final mycelial yield (at day 24) was greater at 25°C than 37°C (Fig. 1).

Antigenicity.

Culture filtrate. The CIE profiles of culture filtrate at day 24 are shown in Fig. 2 and the number of CIE peaks (representing the number of antigenic proteins) in extracts harvested on the different days are given in Table 1. Total number of antigens increased with colony age and as the pH rose. A significantly greater number of antigenic peaks were observed during incubation at 37°C,

than at 25°C, $p=0.01$. Several proteins formed precipitins with patient sera when an intermediate gel incorporating pooled ABPA sera was used (Fig. 2). With the 25°C extract the only precipitin formed within the intermediate gel was completely absorbed, its position in the control main gel is marked with an arrow. Using an intermediate gel containing antisera to Ag7 this peak was identified as Ag7. In the 37°C extract three precipitins that formed lines of identity with peaks in the main gel (marked with arrows) were seen plus an additional peak completely absorbed in the intermediate gel (marked with an asterisk). Use of a monospecific antiserum in the intermediate gel indicated that one of the antigenic peaks (marked Ag7) corresponded to Ag7.

Levels of Ag7 in these cultures are shown in Table 2. The trend was for Ag7 yields to be greater at 37°C than at 25°C except for isolate C on day 14 but the results were variable and did not reach significance. Ag5 was only present in the culture filtrate early (day 2) in incubation; Ag13 was not detected.

Mycelium. The range of antigens detected by CIE of the mycelium extract was smaller than in the extracellular extract (Fig. 3). The number of antigens increased during incubation (Table 1). Similar numbers of antigens were seen at the two temperatures but the proportion of individual components differed greatly. Ag7 levels in the mycelium (Table 2) increased with period of incubation and were significantly higher at 37°C than at 25°C ($P<0.02$).

The effect of culture type on growth and antigenicity

Growth. Agitated culture promoted submerged somatic mycelial growth, while static culture promoted surface sporulation and associated pigment production. Final mycelial mass was greater in agitated culture (49 g) than in

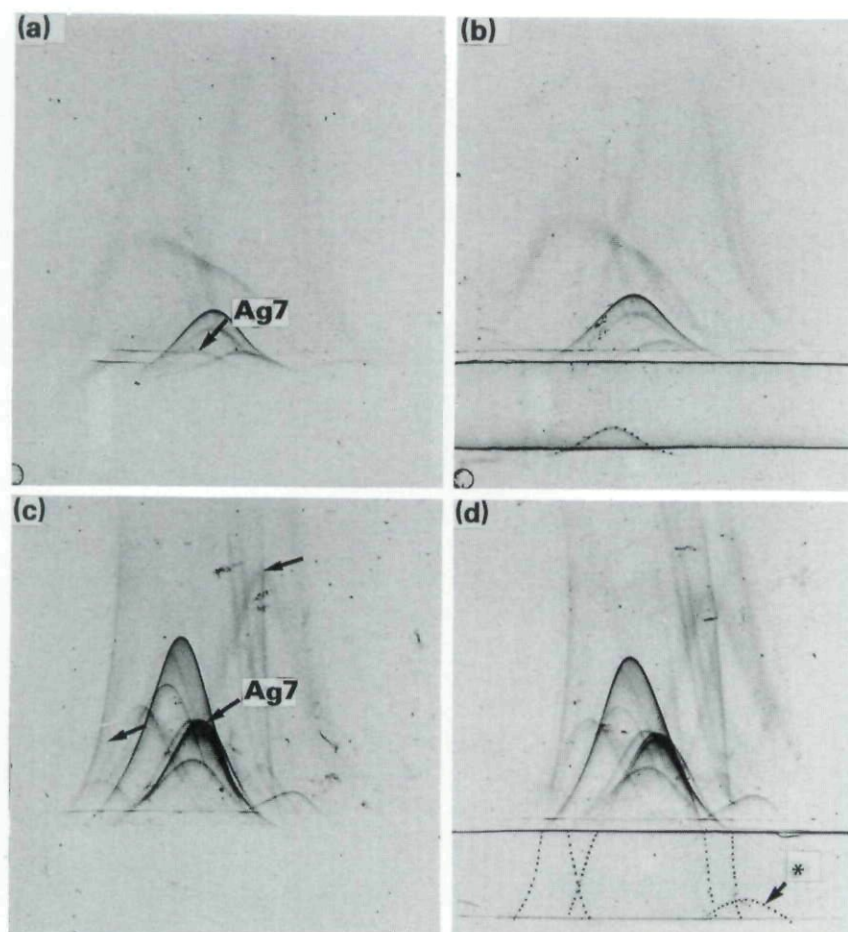


Fig. 2. Crossed immunoelectrophoresis of *A. fumigatus* culture filtrate extracts (3 μ l) isolate a harvested after 24 days incubation at (a) and (b) 25°C; (c) and (d) 37°C. Main gel: hyperimmune rabbit antisera (11.5 μ l/cm²). Intermediate gel: (a) and (c) no serum (control); (b) and (d) ABPA pool serum (20 μ l/cm²). Arrows show peaks in the main gel showing lines of identity with precipitins in the intermediate gel. * Indicates peak not identified in the main gel. Ag7 indicates Antigen 7 peak.

static culture (11 g) after 28 days incubation. The pH changes of the medium of both culture types followed the same trend as described in Fig. 1.

Antigenicity.

Culture filtrate. Total final antigenicity illustrated by CIE is shown in Fig. 4. The culture filtrate antigen range

of the static culture was much greater than that of the agitated culture at days 9 and 28 (Table 3). Five precipitins formed with ABPA pooled serum in the 28 day static culture filtrate (three formed lines of identity with peaks in the main gel area and two were completely absorbed by the ABPA serum) and three in the shake culture. Five allergenic peaks were detected in the static

Table 1. Number of precipitin peaks by crossed immunoelectrophoresis showing total antigenicity of extracts of three isolates (a, b and c) cultured at 37°C and 25°C

Length of incubation (days)	Number of precipitin peaks											
	Culture filtrate ($P=0.01$)						Mycelium ($P=0.04$)					
	25°C			37°C			25°C			37°C		
	a	b	c	a	b	c	a	b	c	a	b	c
2	0	0	0	2	4	0	0	0	0	1	1	0
6	4	3	3	7	5	2	4	2	0	5	3	3
14	7	7	14	19	10	18	5	3	5	7	4	4
24	14	22	>30	>30	>30	>30	9	10	4	11	11	5

Table 2. Levels of antigen 7 in extracts of three isolates (a, b and c) cultured at 25°C and 37°C, height of peak in rocket immunoelectrophoresis

Length of incubation (days)	Antigen 7 (height of peak) in three isolates											
	Culture filtrate (n.s.)						Mycelium ($P=0.018$)					
	25°C			37°C			25°C			37°C		
	a	b	c	a	b	c	a	b	c	a	b	c
2	0	0	0	0	1	0	1	0	1	3	7	2
6	0	1	0	8	0	0	3	7	4	3	14	3
14	2	0	31	25	2	3	7	3	8	<35	8	11
24	5	25	13	21	<35	<35	20	8	23	<35	25	<35

culture filtrate by CRIE using ABPA pool sera and two antigen peaks plus an additional peak not visible with the protein stain were detected in the shake culture filtrate. The peaks are marked by arrows in Fig. 4. Ag7 (marked in Fig. 4) was identified as both allergenic and antigenic.

Rocket immunoelectrophoresis showed that levels of Ag7 were high in both extracts, Ag5 was detected in small quantities in shake culture but was detected more frequently and at higher levels in static culture, Ag13 was only detected in static culture (Table 3).

Mycelium. The antigenicity of the mycelial extract was again more restricted than that of the culture filtrate. Ag7 levels were greater in the mycelium of static cultures than in that of shake cultures (Table 3).

RAST

The extract with the widest antigen range (culture filtrate after 28 days static culture) was used in the RAST. Our RAST detected a higher proportion (30%) of CF patients

with elevated IgE to *A. fumigatus* than the commercial RAST (19%). The usefulness of the assay for diagnosis of ABPA was assessed. The sensitivity of our RAST (92%) was higher than the commercial RAST (72%). However, the specificity of our RAST (84.3%) was lower than the commercial RAST (93.5%). The overall efficacy of our RAST was 88.2% and the commercial RAST was 82.8%.

Discussion

The increase in release of antigens into the medium with the rise in pH during the latter stages of growth of *A. fumigatus* cultures, is similar to that reported by Harvey and Longbottom [7] and Kauffman *et al.* [8]. Many antigens, such as Ag7 and Ag13 are thought to be intracellular cytoplasmic components, only released in appreciable amounts by disruption of the hyphae either by mechanical means or autolysis. The increase in culture filtrate antigenicity found at 37°C may result from greater hyphal lysis or from increased antigen production. The

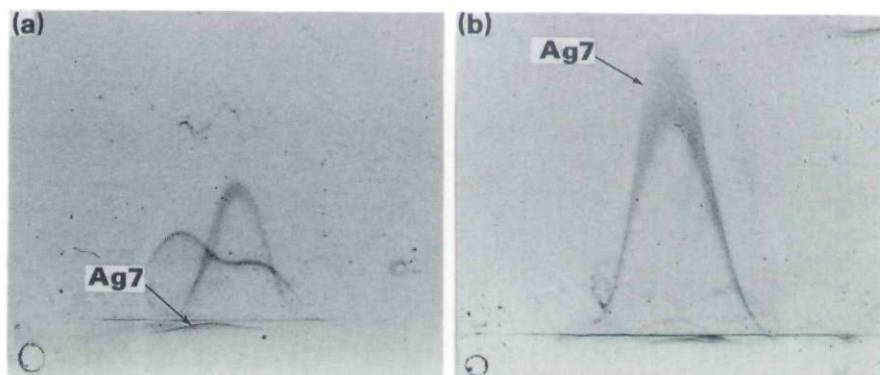


Fig. 3. Crossed immunoelectrophoresis of mycelial extracts (3 μ l) isolate a harvested after 24 days incubation at (a) 25°C; and (b) 37°C; electrophoresed against hyperimmune rabbit antiserum (11.5 μ l/cm²). The Antigen 7 peak is marked (Ag7).

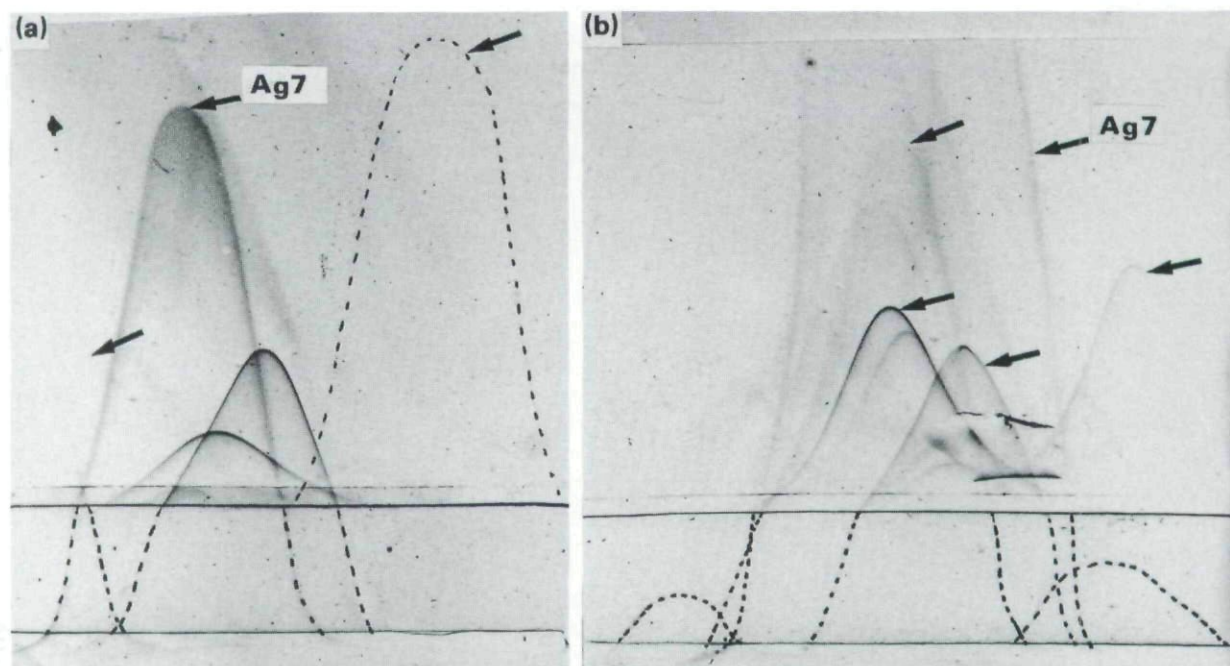


Fig. 4. Crossed immunoelectrophoresis of *A. fumigatus* culture filtrate extracts (3 μ l) after 24 days incubation under (a) shake and (b) static culture conditions. Main gel: hyperimmune rabbit antisera (11.5 μ l/cm²). Intermediate gel: ABPA sera (20 μ l/cm²). The faint precipitin lines have been highlighted with a dotted line, three and five precipitins were formed with patient sera by shake and static extracts respectively. The peaks bound by specific IgE from pooled ABPA sera in CRIE are indicated with arrows (shake three, static five). Ag7 indicates the Antigen 7 peak.

total number of peaks observed with mycelial extracts was similar at the two different temperatures. However, the proportions of individual components were altered, with Ag7 being a major antigen at 37°C but not at 25°C. This suggests that incubation temperature not only promotes release of antigens, such as Ag7, but also promotes the synthesis of these proteins.

The low antigenicity of culture filtrate and mycelial extracts during the early glycolytic phase of growth followed by the production and release of antigens in the later stages of growth, when nutrient depletion is expected to have occurred, suggests that the majority of antigenic proteins could be secondary metabolites.

Kauffman *et al.* [9] reported that certain IgE and IgG

Table 3. The number of precipitin peaks by CIE and levels of Antigen 7, 5 and 13 by rocket immunoelectrophoresis of extracts of shake and static cultures

Extract	Assay	Incubation (days)			
		9		28	
		Shake	Static	Shake	Static
Culture filtrate	No. of peaks	8	11	12	30
	Antigen7 (mm)	0	0	35+	35++
	Antigen5 (mm)	0	11	5	35+
	Antigen13 (mm)	0	0	0	6
Mycelium	No. of peaks	10	9	9	12
	Antigen7 (mm)	4	35+	33	35++

binding components were released exclusively during the early growth phase of shake cultures and our results show some agreement. In this study it was noted that Ag5, the conidial antigen, was only regularly detected during the early stages of incubation of shake cultures. This antigen binds both specific IgG and IgE of ABPA sera [11].

The two different types of culture (static and shake) resulted in different antigenic profiles. Despite the profuse somatic growth observed in shake culture, the total antigenicity of the culture filtrate was less than in the static culture. The heavily sporulating pigmented growth in static culture resulted in greater accumulation of the specific antigens studied and the proteins reacting with patient IgG and IgE in both mycelium and culture filtrate compared with the shake culture. However, the dormant conidia of *A. fumigatus* have been shown to be antigenically inert and only a few antigens are released by mechanical disruption [10] or by germination [11]. A small percentage of conidia were observed to germinate in the liquid culture. However, the large quantity of antigens present in the culture filtrates indicates another source. It is possible that the conidiogenous hyphae associated with conidial production may be involved with the increased antigen production.

This paper illustrates the importance of the incubation methods of *A. fumigatus* on the antigenicity of extracts. Some culture conditions may result in a limited expression of antigenicity and exclude components which may be of importance, especially during the early stages of ABPA, and hence give misleading results in serological diagnosis. This was demonstrated by 25°C cultures compared with 37°C. Patients with cystic fibrosis and ABPA are exposed to *A. fumigatus* colonizing the bronchial airway, and the physiology of the 37°C cultures may consequently reflect the condition of those in the human lung at the same temperature better than the 25°C cultures. The static culture technique increased antigenic diversity and the levels of specific antigens detected (Antigens 7, 13 and 5) and two additional patient IgG and one IgE binding peak were observed. This study indicates that the widest antigenic/allergenic diversity is obtained from the culture filtrate of static cultures at 37°C harvested after 24–28 days incubation. The wide allergenic range was further demonstrated by incorporating this antigen extract in a RAST, which resulted in an assay with increased sensitivity for elevated *A. fumigatus* specific IgE and ABPA when compared with the commercial RAST. The quality of results of allergy tests hinges primarily on the quality of the allergen extracts [16]. Extracts with a broad antigen range should prove useful in screening patients for raised antibody titres to *A. fumigatus* and also provide a useful source for the identification and purification of individual antigenic/allergenic proteins.

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