

Release of antigens and allergens during shake-culture of *Aspergillus fumigatus*

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The appearance of IgG and IgE binding components in the medium of shake cultures of *Aspergillus fumigatus* has been studied. Cultures were grown in synthetic asparagine medium at 35°C and flasks harvested in duplicate 1, 2, 3, 4, 7 and 14 days after inoculation. The pH of the medium dropped from its initial value of 5.5 to pH 3, and then after 4 days gradually increased up to pH 7.5 in the 14-day medium. The weight of mycelium, after an initial peak followed by a slight decline, increased as the pH of the medium increased. Components able to bind IgG and IgE from pooled ABPA sera were detected by crossed immunoelectrophoresis/self-crossed radioimmunoelectrophoresis within 24 h of growth, but maximal release of both antigens and allergens coincided with the increase in pH of the medium and was seen in the 14-day culture filtrate. Two recognised "major" antigens, Ag 7 and Ag 13, detected using the relevant monospecific antisera, were present in the culture medium after 14 days of growth and similarly for Ag 3, the major allergen, although another allergen, Ag 1, was identified in the 1-day extract. None of the culture filtrates was found to contain the "C-substance" polysaccharide.

Key words: antigens; allergens; *Aspergillus fumigatus*; shake culture.

Accepted for publication 4 January 1987

Culture filtrates from long-term (4-5 week) stationary cultures of *Aspergillus fumigatus* are considered to represent the full spectrum of possible antigens and allergens likely to be encountered *in vivo*, but little is known of either the characteristics of release of components or of their relationship with the growth phases of fungus.

Studies on the spontaneous release of antigens and allergens from spores failed to detect any components with immunological activity (4), although rapidly released IgG and IgE binding components from shake cultures have been observed using a microtiter plate binding assay (5, 7). Three phases of growth of the fungus in liquid culture have been reported (3) identified by changes in the pH of the medium, and release of components would appear to be a pH-dependent process. Further information on the antigenic and allergenic composition of the fungus and their relationship with the growth of

the organism, would be useful for standardisation of extracts.

The aim of the present study was to analyse culture filtrates from short-term shake cultures by crossed and self-crossed radioimmunoelectrophoresis (XIE & self-XRIE) for components able to bind to IgG and IgE from allergic bronchopulmonary aspergillosis (ABPA) sera, and to identify the various components, with respect to a standard reference pattern of reactivity obtained using a culture filtrate from a long-term stationary culture (11).

The time of appearance of three major components was also studied using monospecific antisera to the two recognised major antigens of this system, i.e. Ag 7 and Ag 13, and a patient's serum (with known high antibody titre) for the major allergen, Ag 3.

A further component present in extracts from 6-week surface cultures had previously been

shown to behave like pneumococcal C-substance in that it reacted with C-reactive protein (9, 12). The time of appearance of this "C-substance" in short-term shake cultures was studied in relation to the pH changes of the medium and to the release of major antigens and allergens.

MATERIAL AND METHODS

Growth of Aspergillus fumigatus was carried out in 2-litre conical flasks containing 500 ml of sterile asparagine-containing (fully dialysable) medium (13). Flasks were inoculated with 1 ml of a suspension of *A. fumigatus* spores (IMI 16030) (in 0.05% Tween 80) to a final concentration of 2×10^5 spores/ml.

Cultures were incubated at 35°C with vigorous rotation and flasks were removed in duplicate in intervals of 1, 2, 3, 4, 7 and 14 days after inoculation. The pH of the medium was monitored and the pressed wet weight of the mycelium measured.

Extraction of antigens. The culture medium, separated from the mycelium by filtration, was Seitz filtered, dialysed extensively against distilled water and freeze-dried.

Rabbit antisera were raised in New Zealand White and New Zealand White-Dutch lop cross-bred rabbits using culture filtrates from long-term (4–5 weeks) stationary cultures of *A. fumigatus* as previously described (1). High-titre antisera from several bleeds from five rabbits were pooled and used as a reference antiserum.

Monospecific antisera were produced as previously described (1). Briefly, the relevant immunoprecipitate from several identical XIE plates was used as immunogen and the specificity of resulting antisera was confirmed by XIE with intermediate gel.

Human sera. A pool of ABPA serum was formed with sera from 15 ABPA patients (11).

Crossed immunoelectrophoresis (XIE) was as previously described (10). Briefly, glass plates (8 ×

8 cm) were coated with 1% agarose in veronal buffer, pH 8.6. Antigen was applied to a sample well and electrophoresed 4.5 V/cm for 90 min and then rabbit antiserum was incorporated into the antibody-containing gel for the second dimension. Electrophoresis (1.5 V/cm, overnight) was at 90°C to the direction of the first dimension. Plates were washed in 0.9% saline, dried and stained with Coomassie R250.

Rocket immunoelectrophoresis (RIE). Antigen was placed in sample wells 1 cm apart and electrophoresed directly into the antibody-containing gel (1.5 V/cm overnight).

Self-crossed radioimmunoelectrophoresis (self-XRIE) was performed as XIE except that pooled ABPA serum was incorporated into the antibody-containing gel of the second dimension. After washing, the plates were cold air-dried and overlaid with pooled ABPA serum, diluted 1 in 10 in phosphate buffered saline (3 ml) pH 7.4 (PBS). Following overnight incubation the plates were washed 4–5 times with PBS; a second overnight incubation was performed with ^{125}I anti-IgE (Pharmacia) (in PBS/0.3% HSA) and a total of 3×10^5 cpm in 3 ml buffer applied to each plate. The plate was washed 5 or 6 times with PBS, dried and placed against X-ray film (Kodak, DEF2 film) for 7 days. The film was developed according to manufacturers' instructions and the plate stained for protein with Coomassie R250.

Double diffusion tests were performed in 1% agarose (10 ml) on glass slides (8 × 8 cm). A large central well was cut for serum which was applied 2 h before antigen. The antigens were applied to six peripheral wells at a distance of 1 cm from the centre and tests were incubated at 20°C for 2 days. Two identical plates were set up, one was then washed in saline, the other in 5% Na citrate. Both plates were warm air-dried and stained for protein with Coomassie R250.

RESULTS

Fig. 1 shows the results of monitoring (in duplicate) the pH of the medium at various time

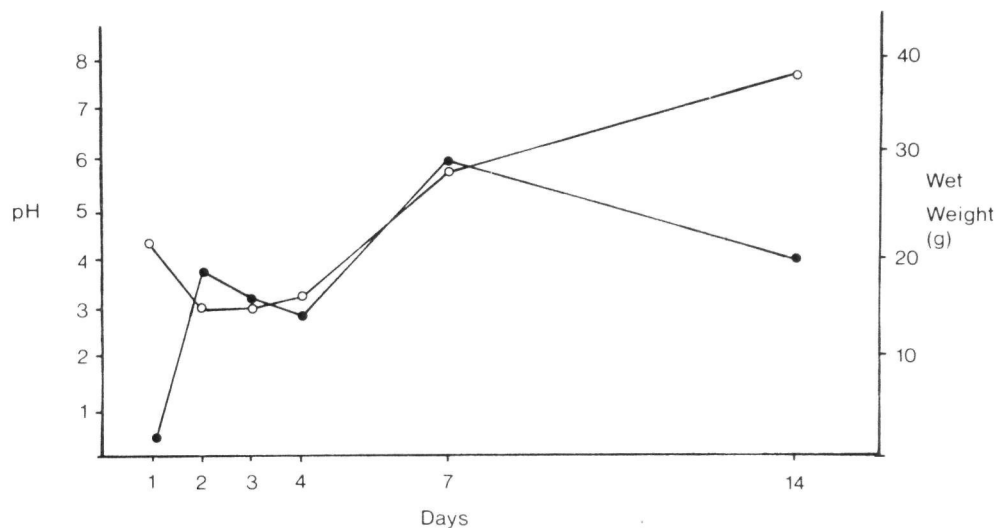


Fig. 1. Changes during growth of *A. fumigatus* in shake culture of the pH of culture medium (○) and wet weight of mycelium (●).

points, together with the changes in the pressed wet weight of mycelium. The pH dropped from its initial value of pH 5.5 to pH 3 after 2 days and then increased again from day 4, up to pH 7.5 in the 14-day medium. The weight of mycelium, after an initial peak at day 2, declined slightly and then increased as the pH increased.

Antigens were detected by XIE tests with rabbit antiserum in all the culture filtrate extracts. Fig. 2 shows the stained XIE plates from the 1, 3, 7 and 14-day culture filtrates. The 1–4 day extracts contained few antigens and most release occurred between 7 and 14 days, and was maximal in the 14-day preparation.

Rocket immunoelectrophoresis using mono-specific antisera to the two major antigens (Ag 7 and Ag 13) showed that trace amounts of Ag 7 were detected in the 1–4 day extracts, but most release of this component occurred between 7

and 14 days. Ag 13 was not visible by RIE before 14 days (Table 1).

Results of self-XRIE of the 1, 3, 7, and 14-day extracts (Fig. 3) showed that IgE binding components were released into the medium within 24 h following inoculation. Maximal release of allergens was observed in the 14-day culture filtrate. Ag 3, the major allergen, was not detected before 14 days.

Culture filtrates from short-term shake cultures were negative by double diffusion with sera from two individuals shown to contain C-reactive protein. Extracts from two long-term (6–8 weeks) stationary cultures known to contain “C-substance” were included as positive controls.

DISCUSSION

Extracts of *A. fumigatus* are complex mixtures of protein and polysaccharide and, in order to standardise preparations, information is required on the growth phases of the organism and the associated antigenic characteristics.

Changes in the pH of the culture medium from the short-term shake cultures were observed, and the pattern followed that previously described (3, 8, 9, 15). After an initial drop in pH, the medium became more neutral, and then alkaline, as the weight of mycelium slowly increased. Fully synthetic asparagine medium

Table 1

Appearance of major antigens and major allergen in culture filtrate of shake cultures determined by rocket immunoelectrophoresis

	1D	3D	7D	14D
Ag 7	-	±	+	+++
Ag 13	-	-	-	+
Ag 3	-	-	±	++

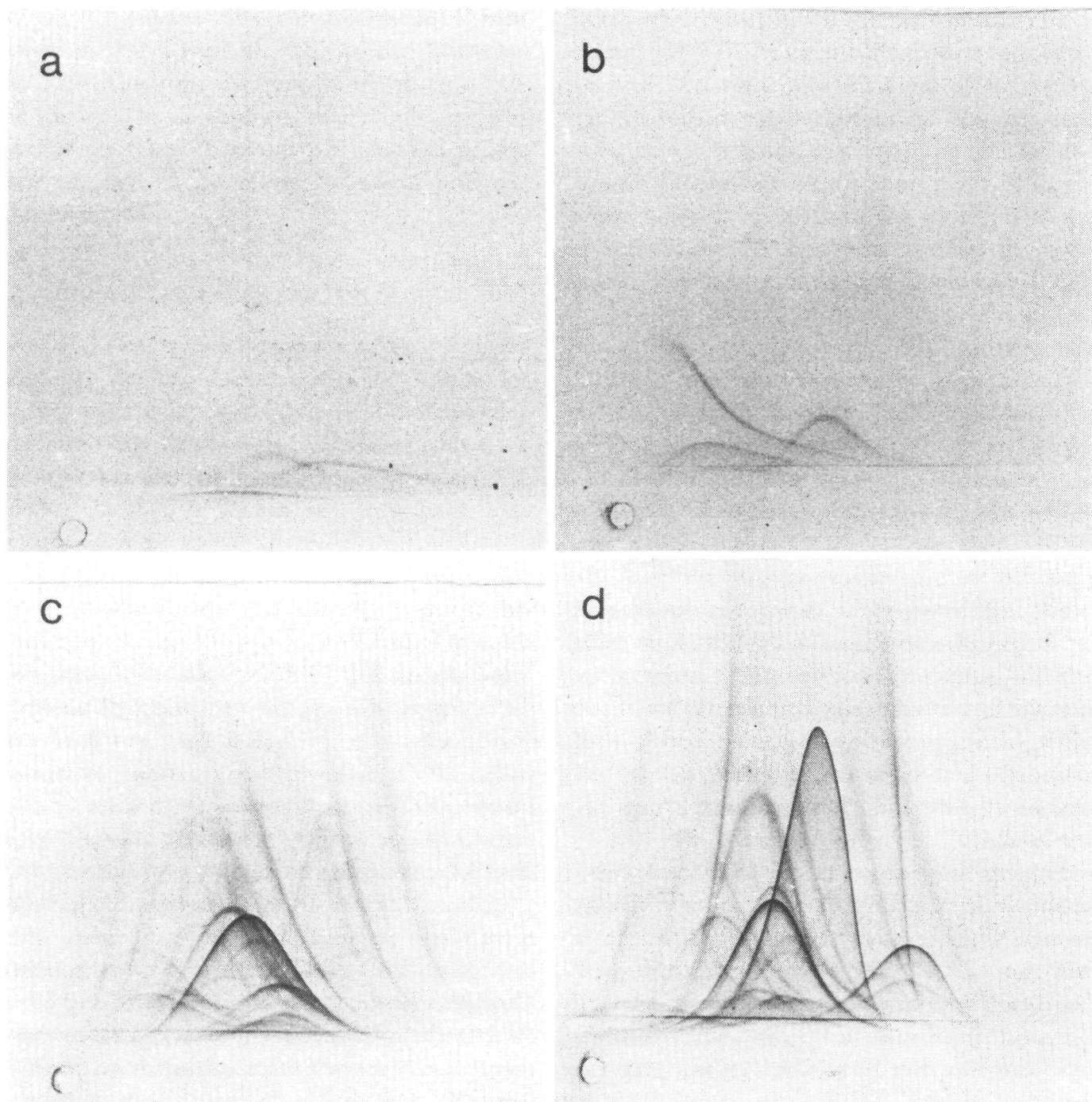


Fig. 2. Crossed immunoelectrophoresis of (a) 1 day, (b) 3 day, (c) 7 day and (d) 14 day culture filtrates (180 μ g antigen electrophoresed into 11 μ l rabbit antiserum/cm² gel).

was used and all three growth phases were observed without the addition of yeast extract which is required when using Czapek Dox (14).

Most antigen released, as determined by XIE with rabbit antiserum and pooled ABPA serum, was associated with the higher pH of the medium, thought to be a result of lytic processes. Results of repeat experiments were found to be qualitatively reproducible when examined by XIE. Antigens released from the growing organism, which may not be present in the culture

filtrate from the long-term stationary cultures used for production of rabbit antiserum, would be detected by autoradiography in self-XRIE, provided the pooled ABPA serum contained specific IgG antibodies.

The major antigen, Ag 7, a high molecular weight glycoprotein, shown to react with specific IgG antibodies from the sera of up to 98% ABPA patients (2, 11) was not detected in appreciable quantities before 7 days, suggesting that it was released due to autolysis. Similarly,

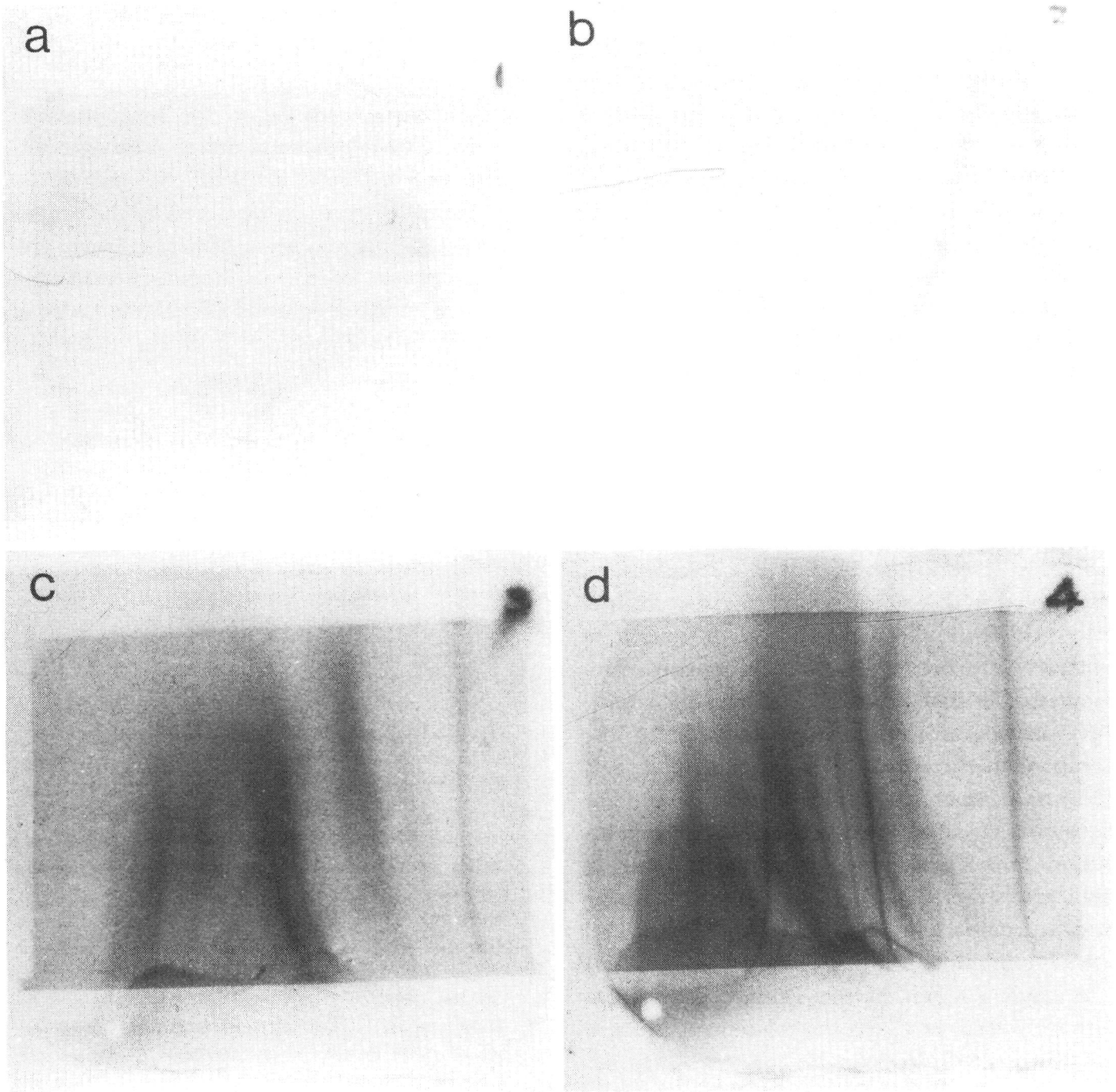


Fig. 3. Autoradiographs from self-crossed radioimmuno-electrophoresis of (a) 1 day, (b) 3 day, (c) 7 day and (d) 14 day culture filtrates ($180\ \mu\text{g}$ antigen electrophoresed into $23\ \mu\text{l}$ pooled ABPA serum/ cm^2 gel).

the other major antigen, Ag 13 (11), identified as the C antigen (1) would also appear to be released into the culture medium as a result of autolysis.

Components able to bind specific IgE from the sera of ABPA patients were detected by self-XRIE after 24 h of growth, and one of these allergens was identified from the standard reference pattern (11) as the fast migrating Ag 1. The major allergen, Ag 3, like the major antigens, was only detected in extracts of the organ-

ism assumed to be in the lytic phase of growth. In another study (5), IgE binding activity, using an enzyme allergosorbent test (EAST), was detected at 4 days, this being the first growth point to be tested.

EAST inhibition was also used in the study of release of IgE binding components (5), with ELISA for IgG binding components (7) and the presence of both these types of components was found to vary with the age and growth phase of the culture. Although rapid (4 day) release was

observed, components disappeared in phase II whilst other additional antigens and allergens were released in phase III (12–30 days), the lytic phase. This contrasted to the present study, where no such variation was detected, but rather a gradual increase in numbers and titres of components was observed during the three growth phases.

The technique of self-crossed radioimmuno-electrophoresis would appear to offer certain advantages over the EAST, EAST inhibition and ELISA techniques as used by other groups (5, 6, 7) since these are influenced by varying numbers and changing concentrations of components competing for binding sites on the solid phase. Self-XRIE does not have a solid phase support, and is not affected by different binding affinities of constituents, but is dependent on the pooled ABPA serum containing specific IgG and IgE antibodies and that both classes of antibody are bound by the components. Previous studies had indicated that for ABPA sera, IgG antibodies were present to most of the components to which there was specific IgE (11), and hence our choice of this method for detection of allergenic components. The sensitivity of the autoradiography is also considerably greater than that of the protein staining and is comparable with that of ELISA and EAST inhibition.

A single allergen, present in the 3-day culture filtrate, was absent from the reference extract, a long-term (4–5 weeks) stationary culture filtrate, though possibly due to the different growing conditions, it was never present in the reference extract.

The “C-substance” type polysaccharide which reacts with C-reactive protein, was not detected in any of the extracts from the short-term shake cultures and is perhaps not an immediate product of lysis. It has been detected in extracts from long-term stationary cultures at 37°C after 5 weeks of growth when the pH of the medium increases to pH 8 or greater and therefore may only be released in later stages of autolysis or where the medium becomes exhausted (9, 12).

These results indicate that whilst both IgG and IgE binding components are rapidly re-

leased from the growing fungus, in order to ensure that culture filtrate extracts contain the major antigens and allergens, i.e. Ags 3, 7, and 13, the fungus must be in the lytic phase of growth. Although the experimental growth conditions are unlikely to bear any degree of resemblance to those encountered by the fungus in the lung, it would seem advisable when preparing extracts for use in diagnostic tests that they be taken from cultures after the increase in pH of the growth medium, however, older cultures may contain “C-substance”.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. P. G. Mantle of Imperial College for help with culturing the fungus, and also Miss M. L. Taylor for technical assistance. This study was supported by the Medical Research Council.

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