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Characterization of a major antigenic component of *Aspergillus fumigatus*

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SUMMARY

A component of *Aspergillus fumigatus*, Ag 7, previously identified as a major antigen for patients with allergic bronchopulmonary aspergillosis, has been isolated by gel filtration and further purified by affinity chromatography using monospecific antiserum. The antigen, which binds both specific IgG and IgE antibodies, was shown to be a high molecular weight, 150–200 kD, heat-stable glycoprotein, which binds to concanavalin A, suggesting the presence of α-D mannopyrannoside, or α-D glucopyrannoside end residues. On sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) it had a subunit of molecular weight 36 kD (with or without prior reduction), which retained antigenicity and allergenicity when tested with patients' sera.

Keywords allergic bronchopulmonary aspergillosis (ABPA) *Aspergillus fumigatus* characterization major antigen

INTRODUCTION

Fungal extracts are complex mixtures of antigens, some allergenic, with relatively little known about the physical and chemical characteristics of individual components and their role in the pathologenesis of disease.

A number of attempts have been made to identify specific antigens of *Aspergillus fumigatus*. An antigen with chymotryptic activity (C antigen) reacting with 53% of sera from patients with aspergillosis was reported (Dessaint *et al.*, 1976). A more recent study described an acidic glycoprotein which reacts with 75% of sera from patients with aspergilloma and allergic bronchopulmonary aspergillosis (Calvanico *et al.*, 1981).

Patients with allergic bronchopulmonary aspergillosis (ABPA) have both specific IgG and IgE antibodies to *A. fumigatus*. Using a modified technique of crossed radioimmunoelectrophoresis (XRIE) in which patient's serum was incorporated into the antibody-containing gel of the second dimension (self-XRIE), and comparing individual responses to a pooled ABPA 'reference' serum, two types of antigenic components were identified, those poorly precipitating but showing strong IgE binding, and those producing the strongest precipitin reaction with only weak IgE binding (Longbottom, 1983). Of these latter components two in particular, Ag 7 and Ag 13, reacted with over 70% of the sera from ABPA patients, and were regarded as being 'major' antigens.

The aim of this study was therefore to purify and characterize one of these major antigens, Ag 7.

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MATERIALS AND METHODS

A. fumigatus antigen. Batches of the fungus were grown as long-term (4–5 weeks) stationary cultures on an asparagine synthetic medium (Smith *et al.*, 1948) at 25°C. The culture filtrate (cf) was separated from the mycelial mat, filtered, dialysed and freeze-dried.

Mycelial extracts (myc) were prepared by sonication (MSE Soniprep) of homogenized mycelium in Coca's solution at 4°C, by repeated 5 min bursts (maximum power) until > 80% of hyphae were disrupted. This procedure was followed by filtration, dialysis and freeze drying.

Salt precipitation. Crude cf extract was dissolved in water to a final concentration of 30–50 mg/ml, and an equal volume of saturated ammonium sulfate was slowly added with stirring. Solid ammonium sulfate ($(NH_4)_2SO_4$) was gradually added until saturation was reached. After standing at room temperature overnight, the precipitate was removed by centrifugation, washed twice with saturated $(NH_4)_2SO_4$ and resuspended in distilled water. The protein-enriched precipitate was extensively dialysed and freeze-dried.

Human sera. A reference ABPA serum pool was prepared as previously described (Longbottom, 1983).

Rabbit antisera. Antisera to culture filtrate extracts were raised in New Zealand White and New Zealand White-Dutch lop cross-bred rabbits. A 10 mg extract in 2 ml saline was emulsified with Freund's incomplete adjuvant and injected subcutaneously into multiple sites. This was repeated after 4 weeks and followed by intramuscular injections in saline every 4–6 weeks until a satisfactory response was obtained, as determined in XIE tests. High titre antisera from several bleeds from five rabbits were then pooled and used as a reference antiserum.

Monospecific antisera. The relevant precipitin peak excised from a number of duplicate XIE plates (as seen in Fig. 2a) was emulsified in Freund's incomplete adjuvant or merely sonicated with saline for boost injections (Hornsleth, Mordhorst & Pedersen, 1980; Hansen, Vestergaard & Hansen, 1981). The immunization procedure was as for polyspecific rabbit antiserum.

Crossed immuno- and crossed radioimmuno-electrophoresis (XIE/XRIE). XIE and XRIE were performed as previously described (Longbottom, 1978). In some XIE and XRIE a second antiserum was placed in an intermediate gel between the sample and the antiserum-containing gel of the second dimension. Such tests were carried out with controls where the antiserum was omitted from the intermediate gel.

Fused rocket immunoelectrophoresis (FRIE). Samples from alternate fractions (following gel filtration) were applied to sample wells and diffused for 1 h before electrophoresis into rabbit antisera.

Gel filtration chromatography. A 500 ml column (C26/100, Pharmacia, Milton Keynes, UK) was equilibrated with Sephacryl S200 (Pharmacia, Milton Keynes, UK) in 0·05 M ammonium hydrogen carbonate (NH_4HCO_3) buffer and calibrated using markers of known molecular weight.

Protein enriched extracts (150 mg) in 5 ml buffer were applied to the column at a flow rate of 25 ml/h and after monitoring spectrophotometrically at 280 nm were collected in 4 ml fractions and further monitored by FRIE.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed in a 15% acrylamide separating gel in 0·125 M Tris, pH 8·8, containing 0·1% SDS. Samples were made up in 0·375 M Tris buffer, pH 6·8, and reduced samples were pretreated with beta-mercaptoethanol, 70°C for 20 min. Gels were stained for protein with Coomassie blue, or carbohydrate using periodic acid/Schiff's (PAS) reagent (Zacharius & Zell, 1969), or were electrophoretically blotted onto nitrocellulose membrane (see below).

Western Blotting. Components separated on SDS-polyacrylamide gels were transferred at 1 mA (~ 0·1 V) overnight onto nitrocellulose immobilizing membrane (Burnette, 1981). Unreacted sites were then blocked by incubation with 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) containing 0·5% Tween-20. After washing the membrane was incubated with serum (diluted 1 in 10 with PBS) overnight, washed with PBS containing 0·5% Tween-20, and then incubated overnight with either ^{125}I-Protein A (Amersham International, Amersham, UK) or ^{125}I-anti-IgE (Pharmacia, Milton Keynes, UK). The nitrocellulose was washed, dried and placed against X-ray

film (Kodak, DEF-2) for 5–7 days, after which the films were developed according to manufacturer's instructions.

Preparation of Ag 7 by affinity chromatography. IgG was extracted from the monospecific antiserum using caprylic acid (Steinbuch & Audran, 1969) and was then covalently bound to CNBr-activated Sepharose 4B (Pharmacia, Milton Keynes, UK) according to manufacturer's instructions. Ten milligrams of IgG were coupled to 1g Sepharose.

The coupled Sepharose was incubated with a partially purified antigen sample (fractions 56–61 from the gel filtration column) 30 mg/ml in PBS, and gently agitated at 4°C overnight. Unbound antigen was removed by washing the Sepharose with PBS on a sintered glass funnel.

Purified Ag 7 was obtained by elution from the Sepharose by gentle mixing in a tube with 10 ml 1 M propionic acid for 5 min, followed by centrifugation. The supernatant was removed and added to an equal volume of 1 M Tris pH 8·4. This process was repeated with a further 10 ml propionic acid followed by 2 × 10 ml PBS. Combined supernatants were extensively dialysed against distilled water (4°C), and freeze-dried.

RESULTS

Gel filtration

Gel filtration of the protein-enriched extracts, as monitored by fused rocket immunoelectrophoresis with rabbit antiserum (Fig. 1a), showed that antigens eluted from the column throughout the molecular weight range, > 200–210 kD. Two main antigen peaks in particular appeared to elute in the initial first peak of OD activity, mol. wt 150–200 kD (fractions 56–61). The presence of two main components was confirmed by XIE of the combined fractions (Fig. 2a) and although results are not shown here, these components were also found to be both antigenic and allergenic for the ABPA sera.

Monospecific antiserum

The outer antigenic peak seen on XIE of the pooled column fractions tested against the polyvalent reference antiserum, is indicated in Fig. 2a. A monospecific antiserum was produced to this component and the reaction of this antiserum with the pooled fractions is shown in Fig. 2b. The specificity of this antiserum for a single component was confirmed by its incorporation into an intermediate gel (Fig. 2c, d).

Identification of component

Incorporation of the monospecific antiserum into an intermediate gel in the standard reference pattern with pooled ABPA sera (Fig. 3), identified the component specifically absorbed as Ag 7 (Longbottom, 1983).

Identification of Ag 7 in extracts

By rocket immunoelectrophoresis using the monospecific antiserum, Ag 7 was detected in all culture filtrate and mycelial extracts (Fig. 4A) and shown to be present in greater amounts on a dry weight basis in the mycelial extracts.

Affinity purification of Ag 7

The yield of purified antigen eluted with propionic acid from the monospecific antiserum-linked affinity column was extremely low. Its purity, as determined by XIE with both the rabbit antiserum and the monospecific antiserum, was found to be high. However, when analysed by SDS-PAGE and Western Blotting there were traces of non-antigenic contaminants with molecular weights of 57 kD and 61 kD.

Physicochemical characteristics of Ag 7

Molecular weight (Sephacryl S200 and SDS-PAGE). Confirmation that Ag 7 eluted from the gel filtration column with a molecular weight of 150–200 kD was obtained by incorporation of

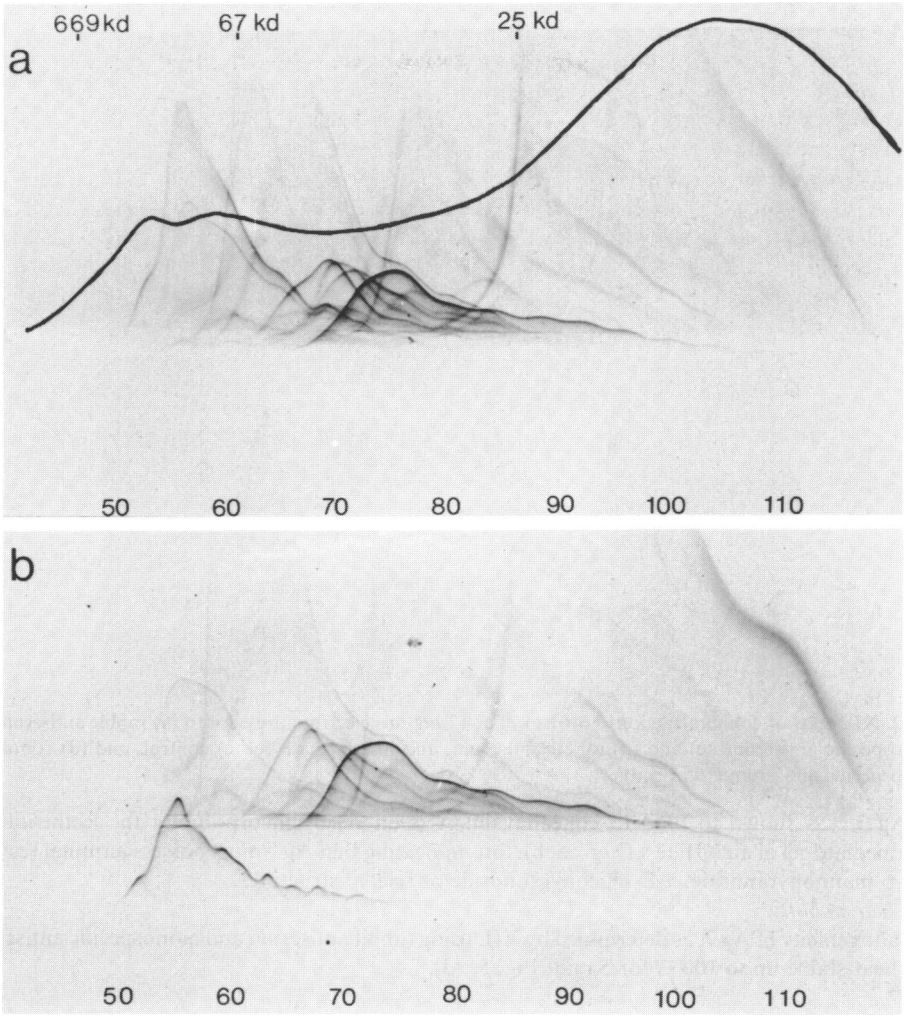

Fig. 1. (a) FRIE profile of Sephacryl S200 separation of protein-enriched culture filtrate. Four μl of alternate fractions were electrophoresed into 9 μl/cm^2 rabbit antiserum. Positions of molecular weight markers are shown, and the optical density (280 nm) of eluate. (b) FRIE profile with monospecific antiserum (10 μl/cm^2) incorporated into intermediate gel.

monospecific antiserum into an intermediate gel in a fused rocket profile of the Sephacryl S200 fractionation (Fig. 1b).

On SDS-PAGE, however, Ag 7 appeared as a single diffuse band with a molecular weight of 36 kD (Fig. 4B) irrespective of whether or not there was prior reduction of the sample with beta-mercaptoethanol.

Incubation of a Western Blot obtained after SDS-PAGE separation of affinity purified Ag 7 with: ABPA (pooled) sera, followed by ^{125}I-anti-IgE; ABPA sera, followed by ^{125}I-Protein A; and monospecific antiserum, followed by ^{125}I-Protein A; showed that the 36 kD band was both allergenic and antigenic for patients' sera, and was antigenically recognized by the monospecific antiserum (results not shown).

Carbohydrate nature of Ag 7. The 36 kD band of Ag 7 on SDS-PAGE stained with periodic acid/Schiff's reagent for carbohydrate and Coomassie blue for protein.

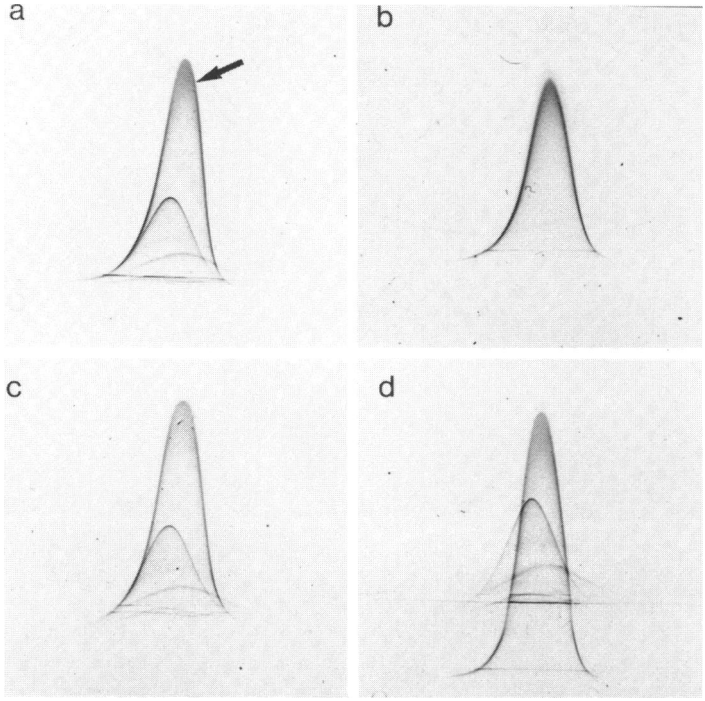

Fig. 2. XIE tests of column fractions 56–61 (3 μl at 12 mg/ml) electrophoresed into (a) rabbit antiserum, (b) monospecific antiserum, (c) and (d) rabbit antiserum, and intermediate gel (c) control, and (d) containing monospecific antiserum (10 μl/cm^2).

Ag 7 was shown to bind to concanavalin A (Con A) by incorporating the lectin into an intermediate gel of an XIE test (Fig. 5a, b), thus indicating that Ag 7 must possess terminal residues of α-D mannopyranoside, α-D glucopyrannoside or related structures.

Heat stability

The antigenicity of Ag 7, as determined by XIE using rabbit antiserum and monospecific antiserum, was heat-stable up to 100°C for 5 min (Fig. 5c, d).

DISCUSSION

Culturing of *Aspergillus fumigatus* for production and extraction of antigens is affected by many variables and even under apparently controlled conditions results are not necessarily reproducible (Kurup *et al.*, 1978). In liquid culture, three phases of growth have been recognized by changes in the pH of the medium (Kauffman & De Vries, 1980). In this study the extracts were all from the third phase of growth (associated with a rise in pH) where the medium is becoming exhausted of nutrients and lysis of the organism is occurring, thereby releasing cell constituents into the medium. Such culture filtrates are thought to contain not only any extracellular components released in early phases of growth, but also all the metabolic, intracellular components, and have therefore been considered to contain a full spectrum of antigens and allergens. It is possible however that, as many enzymes are present, some denaturation can occur.

A. fumigatus extracts contain large proportions of polysaccharide, in particular galactomannans from the cell walls, and although antibodies to those polysaccharides have been demonstrated in human sera they are less specific and tend to cross-react with polysaccharides present in other fungi. Therefore, in this investigation the polysaccharide was removed from the extracts using ammonium sulfate salt precipitation so that all separations and purification procedures were performed on the more specific and highly antigenic, protein-enriched extracts.

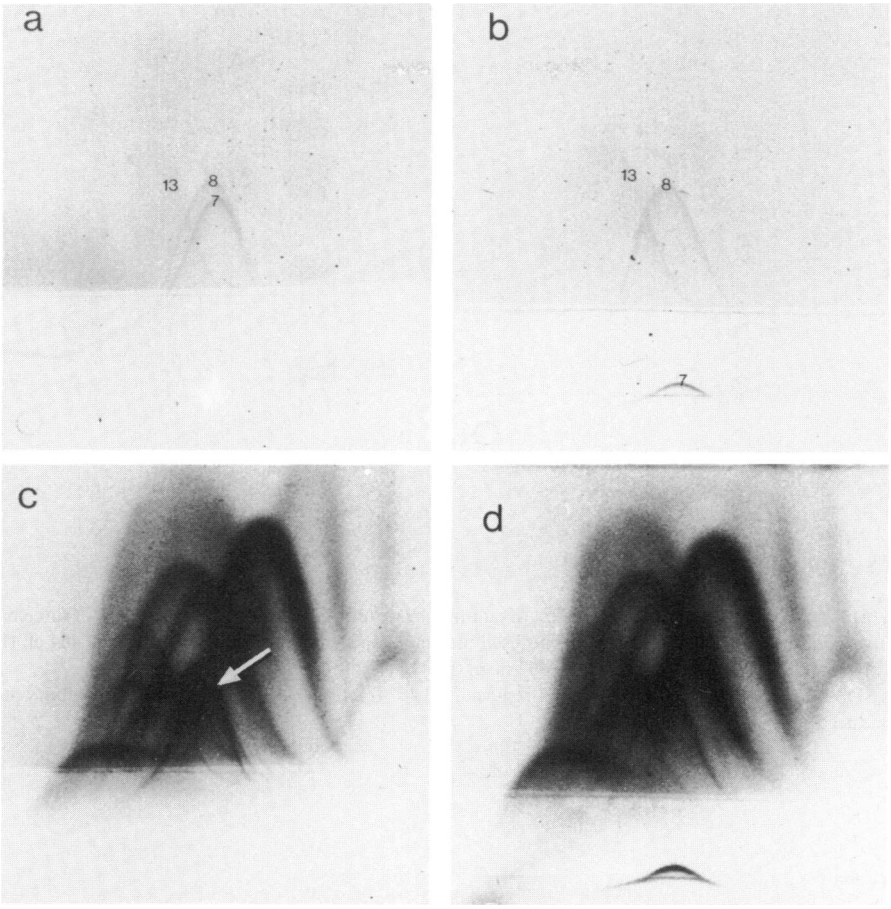

Fig. 3. Self-XRIE tests using the pooled ABPA reference serum. For each test 1·5 μl *A. fumigatus* antigen (B27 at 12 mg/ml) was used with 23 μl/cm² serum. (a) and (b) are the Coomassie stained plates and (c) and (d) the corresponding autoradiographs. An intermediate gel incorporating 10 μl/cm² monospecific antiserum was included in (b) and (d).

Gel filtration chromatography proved a useful step for partial separation of the 30–40 antigenic components in extracts and for the initial purification of Ag 7. Despite the specificity and high affinity of the monospecific antiserum that was raised to Ag 7, affinity chromatography proved relatively unsuccessful for its purification, even though many sets of conditions using different buffers were tried. Reasons for the consistently low yields are not understood.

Ag 7 was identified in all extracts of *A. fumigatus*, but appeared to be present in greater amounts (by dry weight) in mycelial extracts.

Ag 7 was shown to be a heat-stable, acidic glycoprotein, with α-D mannopyrannoside or α-D glucopyrannoside terminal residues. It is a high molecular weight molecule of 150–200 kD, apparently composed of 36 kD subunits. These subunits are not bound by disulfide bridges, but are held together by weak non-covalent forces which were destroyed by the detergent sodium dodecyl sulfate. Both the high molecular weight molecule and its subunits appeared to contain carbohydrate moieties and all antigenic and allergenic activity was retained in the tertiary structure when the quaternary structure of the molecule was destroyed.

Attempts have been made by other investigators to identify and purify important components of *A. fumigatus*. An antigen (C antigen) was identified which possessed chymotryptic activity and

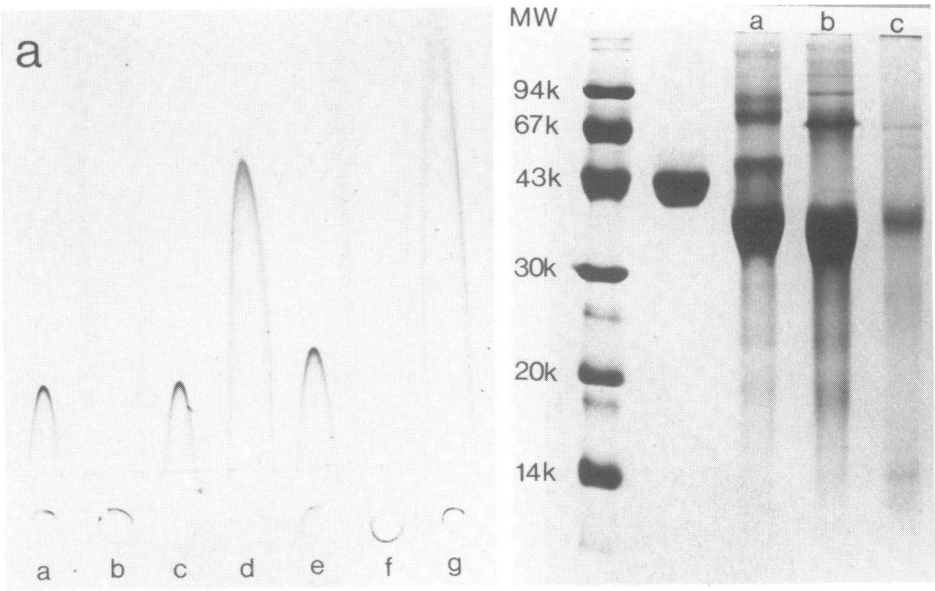

Fig. 4. (a) Rocket immunoelectrophoresis test of culture filtrate (cf) and mycelial (myc) extracts of three batches of *A. fumigatus* (3 μl at 30 mg/ml) electrophoresed into monospecific antiserum (9 μl/cm^2). (a) 464 cf, (b) 464 myc, (c) 465 cf, (d) 465 myc, (e) 467 cf, (f) 467 myc, (g) 475 cf.

(b) SDS-PAGE of (a) 475 fractions 56–61 reduced, (b) 467 fractions 56–61 non-reduced, (c) affinity purified Ag 7 reduced.

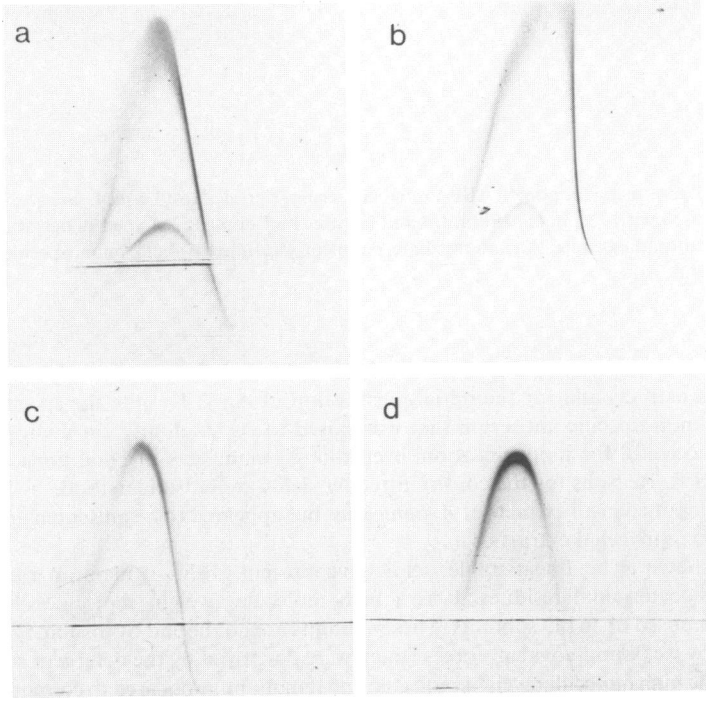

Fig. 5. XIE tests of fractions 56–61 (3 μl at 12 mg/ml) electrophoresed into rabbit antiserum (9 μl/cm^2). Concanavalin A (10 μl/cm^2 at 2 mg/ml) has been incorporated into an intermediate gel (a) shown with control (b). (c) The effect of heating the sample, 56°C for 30 min and (d) the effect of heating at 100°C for 5 min (d), with monospecific antiserum (!0 μl/cm^2) incorporated in an intermediate gel.

reacted with 53% of sera from patients with aspergillosis (Dessaint *et al.*, 1976). A monospecific antiserum to the C antigen (kindly supplied by Drs S. De Magaldi & D.W.R. Mackenzie (1984)) has been used to demonstrate that this antigen is in fact antigen 13 of our reference system (Longbottom, 1983). Although like Ag 7, Ag 13 is regarded as a major antigen, i.e. the majority of ABPA sera possessing IgG antibody, there was considerably less binding of specific IgE to it than occurred to Ag 7.

Highly antigenic, high molecular weight fractions from gel filtration chromatography have also been reported, but all contained more than one component (Schønheyder & Andersen, 1984a). An antigen possessing catalase activity in the fraction with a molecular weight of 250 kD is unlikely to be Ag 7, since no catalase activity could be detected in Ag 7 (personal observation). Ag 7 may be present, however, in the 160 kD antigen fraction which was prepared by hydrophobic interaction chromatography followed by gel filtration, and found to be highly reactive in an enzyme-linked immunosorbent assay (ELISA) system with sera from aspergilloma patients (Schønheyder & Andersen, 1984b).

An acidic glycoprotein, which reacted with 75% of sera from patients of aspergilloma and ABPA, has been partially characterized (Calvanico *et al.*, 1981). This is a cell sap component with a high molecular weight of 150–200 kD by gel filtration, but it is either composed of four 45 kD subunits linked through disulfide bridges, or of three subunits of molecular weights 41, 53 and 80 kD (Piechura *et al.*, 1983). Although Ag 7 has been detected in the preparations from which this cell sap component was extracted (personal observation), further work is required to determine whether these two antigens are the same or related molecules.

Another, cell wall-derived, glycoprotein antigen of *A. fumigatus* also appears to share various characteristics with Ag 7, in that it elutes from gel filtration in a similar molecular weight range (140–300 kD) and binds to concanavalin A (Weiner & Coats-Stephen, 1979). It would be particularly interesting if these were proved to be the same antigen components, since this cell wall antigen was used not to detect specific antibody but in an inhibition radioimmunoassay for the detection of antigenemia in systemic aspergillosis.

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