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Antigens of *Aspergillus fumigatus*

I. PURIFICATION OF A CYTOPLASMIC ANTIGEN REACTIVE WITH SERA OF PATIENTS WITH ASPERGILLUS-RELATED DISEASE

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SUMMARY

An antigen has been purified from the mycelial cell sap of *Aspergillus fumigatus* (strain 507). The same component appears to be present in the extracellular phase (culture filtrate) in a partially degraded form. The cell sap protein has a structure composed of four polypeptides of 45,000 daltons linked through disulphide bonds. The isoelectric point (5·2–5·6) and carbohydrate content (12·5% neutral hexose) indicate that this protein is an acidic glycoprotein. It shows reactions with 75% of sera from patients with aspergilloma and allergic bronchopulmonary aspergillosis and is not reactive with sera from normal individuals or patients with other fungal diseases. It also appears to be a component of other *A. fumigatus* strains.

INTRODUCTION

Aspergillus fumigatus is a common opportunist pathogen with a worldwide distribution. Both allergic and non-immunological disorders can result from exposure. Allergic disorders include atopic respiratory disease (asthma), allergic bronchopulmonary aspergillosis (ABPA) and hypersensitivity pneumonitis (HP). Non-immunological disorders due to Aspergillus include aspergilloma (cavitary aspergillosis) and invasive aspergillosis (systemic aspergillosis) (Pepys, 1978; Pennington, 1980; Schatz, Patterson & Fink, 1979).

Detection of specific precipitating antibody against extracts of *A. fumigatus* in patient sera has been found to be an important aid in the diagnosis of various forms of aspergillosis (Longbottom & Pepys, 1964). Direct comparison of serological data obtained by different investigators, however, is difficult because of the wide variability in reactivity and relatively crude nature of the antigen preparations used (Fink *et al.*, 1977; Malo *et al.*, 1977; Hoehne, Reed & Dickie, 1973). In a previous report from this laboratory (Kurup *et al.*, 1978), 11 selected strains of *A. fumigatus* were studied to determine the effect of culture conditions on culture filtrate (CF) antigen reactivity with known positive sera from patients with Aspergillus-related disease. When used individually, CF preparations from various strains were reactive with 42–87% of the positive sera. The strain showing the highest percentage of reactivity (*A. fumigatus* strain 507) has been selected as a source of antigen for the fractionation and characterization studies reported in the present study.

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MATERIALS AND METHODS

Organism. A. fumigatus strain 507 was isolated in our laboratory from the sputum of a patient with aspergilloma. It was grown on Sabouraud's agar slants and maintained at 4°C. For fermenter inoculation a standard spore suspension of *A. fumigatus* was prepared from a 4-day-old Sabouraud's agar culture. The spore concentration was adjusted to 4×10^6 spores per ml, frozen at −20°C, and used for all subsequent growth. Morphology was confirmed at each step by the criteria of Raper & Fennell (1973).

Culture conditions. A. fumigatus (strain 507) was grown in 10-litre batches in a microfermenter (New Brunswick Scientific, Edison, New Jersey) maintained at 37°C with 3 litres of filtered air per min with constant stirring at 200 r.p.m. The growth medium consisted of a completely defined and dialysable mixture of 50% (v/v) each of AOAC and Czapec-Dox (CD) broth (DIFCO Laboratories, Detroit, Michigan) and 1% glucose (AOAC/CD-G). Thirty million colony-forming units (CFU) were inoculated into the fermenter. Sterile 50% anti-foam (Sigma Chemical Co., St Louis, Missouri) was added as required. Cultures were killed by the addition of formalin to a final concentration of 0·5% for 18 hr.

Growth curve. Preliminary studies were undertaken to determine the optimal fermenter growth period. The criteria used to determine maximal yield were the amounts of mycelia and exocellular material released into the growth medium (culture filtrate).

Culture filtrate material. After 4 days of fermenter growth, the culture was killed and the mycelial portion was separated from the growth medium by filtration through sterile gauze. Non-dialysable components (> 10,000 daltons) were prepared by diafiltration through a micropore membrane (Millipore Corp., Bedford, Massachusetts).

Cell sap material. The mycelial portion of the 4-day fermenter growth was washed with distilled water and frozen (−70°C) until used. Mycelia and 0·2 M phosphate buffer, pH 7·2 (PB), were ground with 0·45 mm glass beads in a Virtis 23 homogenizer (The Virtis Co., Gardiner, New York) and maintained at 2–4°C. The volume of glass beads used was equal to the amount of mycelial suspension. The suspension was made 10 mM in phenylmethylsulphonyl fluoride (PMSF; Eastman Kodak, Rochester, New York) to inhibit proteolytic degradation of proteins. Per cent breakage of mycelia was determined microscopically and was found to vary between 60–85% in separate batches. Cell debris, unbroken cells and glass beads were separated from cytoplasmic (cell sap) material by centrifugation at 48,000 *g* for 1 hr at 4°C in a Sorval R5B refrigerated centrifuge (DuPont Instruments, Newtown, Connecticut).

The supernatant and the first PB wash were concentrated approximately 20 times by positive pressure (PM-10 membrane; Amicon, Bethesda, Maryland) and centrifuged for 5 hr at 105,000 *g* through 15% sucrose buffer [15% sucrose (w/v) in 0·1 M NaCl, 0·03 M $MgCl_2$, 0·01 M Tris–HCl buffer, pH 7·4] (Rubin, 1975). Following centrifugation, the top layer containing lipid material was removed by aspiration and the clear CS material removed from the bottom layer.

Gel filtration. Gel filtration was carried out on $2{\cdot}5 \times 100$ cm columns of Sephadex G-200 (Pharmacia, Piscataway, New Jersey) or Ultrogel AcA 4cA 44 (LKB, Bromma, Sweden) at 4°C. Fractions of 150 drops (~6·5 ml) were eluted with PBS, pH 7·2. Columns were calibrated with normal human serum. Protein content was determined by absorbance at 280 nm. Fractions containing material reactive with patient sera or with rabbit antisera to *A. fumigatus* (strain 507) were located by double diffusion (DD). Fractions were pooled according to their reactions, concentrated by positive pressure and freeze-dried.

Immunodiffusion. Double diffusion in agar gel was carried out by a modification of the method of Ouchterlony (1953). Gels were washed with 0·9% saline and deionized water, dried and stained with 0·25% Coomassie blue G-250.

Polyacrylamide gel electrophoresis. Discontinuous gel electrophoresis was carried out on 5% gels at 3 mA per gel using the method of Davis (1964). Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 7·5% gels following the method of Weber, Pringle & Osborn (1972). Gels were fixed overnight in 10% TCA and stained for protein with Coomassie blue G-250 (Eastman Kodak, Rochester, New York) or stained for carbohydrate with periodic acid–Schiff (PAS) stain (Kapitany & Zebrowski, 1973).

Crossed immunoelectrophoresis (CRIEP). The method used was a modification by Kurup & Fink (1977) of the method of Thirkill & Kenny (1974). Briefly, a 2·5 × 10 cm strip of 1% agarose in barbital buffer (0·05 M, pH 8·6) containing 0·1% sodium azide was poured onto an 8 × 10 cm sheet of GelBond film. Thirty microlitres of sample (1–3 mg per ml protein) were electrophoresed for 1·5 hr at 8–10 V/cm. Following electrophoresis, the second-dimension agarose gel containing 10% rabbit antiserum was poured. The plates were electrophoresed at right-angles to the original direction for 16 hr at 1–2 V/cm. Precipitin lines were allowed to develop overnight in a humid chamber. The plates were stained with Coomassie brilliant blue according to the method of Axelsen & Bock (1972).

Analytical isoelectric focusing. Twenty-microlitre samples containing 15–30 μg of protein were run on prefocused LKB Ampholine PAG plates, pH 3·5–9·5 (Bromma, Sweden), for 1·5 hr at 50 mA. After focusing, the PAG plate was fixed and stained with Coomassie brilliant blue except for one strip parallel to the migration which was divided into 1-cm segments and placed in water overnight. The pH of the individual segments was determined in this manner in order to construct the pH gradient of the gel.

Protein determination. Protein content was determined by the method of Lowry *et al.*, (1951) or by the Coomassie brilliant blue dye-binding method of Bradford (1976). Bovine chymotrypsinogen A (Worthington Biochemical Corp., Freehold, New Jersey) was used as the standard.

Neutral hexose determination. The natural hexose content was determined by the anthrone reaction as described by Roe (1955) using a glucose–galactose standard. Values were expressed as a percentage of dry weight.

Antiserum. Antiserum to *A. fumigatus* (strain 507) was raised in NZW rabbits by repeated subcutaneous injections of mycelia, CF material and spores in Freund's incomplete adjuvant.

Human sera. Human sera from patients with aspergilloma, ABPA, or other fungal diseases, and normal controls were obtained and frozen until used. Informed consent was obtained from all subjects. Ouchterlony analysis of patient's sera with CS2 was carried out by diluting CS2 to an optical density at 280 nm of 1·8 (approximately 0·9 mg/ml by dry weight). This was tested for precipitin formation by filling the wells twice with the patients' serum. This procedure was found to be optimal for the detection of positive reactions and was used as our standard assay. Precipitins were arbitrarily graded from + to + + +, depending on intensity.

A. fumigatus *stationary culture antigens.* Stationary cultures of *A. fumigatus* (strains 507, 534 and 515) were grown for 3 weeks at 37°C in AOAC/CD-G medium. Culture filtrate was exhaustively dialysed against distilled water and then freeze-dried. For routine double-diffusion studies, the antigens were prepared at 10 mg/ml in deionized water.

RESULTS

Growth curve. During the 10-day growth period, the dry weight of mycelia increased steadily up to and including day 4 and then decreased. The weight of non-dialysable culture filtrate material increased steadily up to and including day 9 and then dropped precipitously. Part of the increase in culture filtrate material is probably due to liberated cytoplasmic material as a result of cell death. Because of our interest in both culture filtrate antigens and cell sap antigens, day 4 was selected as optimal under the given growth parameters and was used in further fermenter runs for antigen production.

The protein and neutral hexose content of CF growth curve material gave a biphasic curve. Protein and neutral hexose content (w/w %) increased to a peak of about 40% on day 5, decreased sharply to approximately 15% on day 6, increased to a second peak on day 8, and then decreased slightly on days 9 and 10. On day 4 of culture, the protein content of CF material was 34% and the neutral hexose content was 31% by dry weight (w/w%).

Disc electrophoresis. Disc electrophoresis of CF and CS antigens from fermenter cultures and CF from stationary culture are shown in Fig. 1. Several similarities are seen between the CF obtained from both fermenter-grown and stationary-cultured *A. fumigatus* (strain 507). Both culture filtrates have a major component in the top half of the gels, but most of the components are

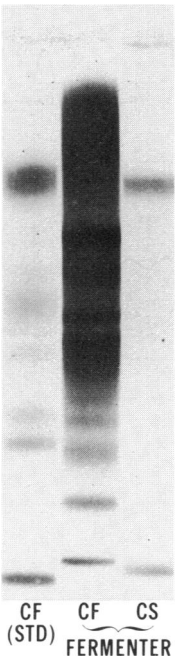

Fig. 1. Disc electrophoresis (running pH of 9·5) on 5% polyacrylamide gels of *A. fumigatus* (strain 507) culture filtrate grown in stationary culture (CF STD) and fermenter-grown culture filtrate (CF) and cell sap extract from mycelial phase (CS). Anode is at bottom of gel.

in the lower half. In addition, the fermenter-grown CF appears to have a considerable amount of background staining indicative of glycoprotein heterogeneity.

In contrast to the CF, CS appears to be a considerably less heterogeneous mixture. One component appears prominent in the upper half of the gel. The lower half appears almost devoid of banding. All bands stained with both PAS and Coomassie blue indicating that they contained glycoproteins.

Double-diffusion reactions. Sera of patients with aspergilloma show a variety of precipitin reactions with stationary CF from various strains of *A. fumigatus* on double-diffusion analysis. The

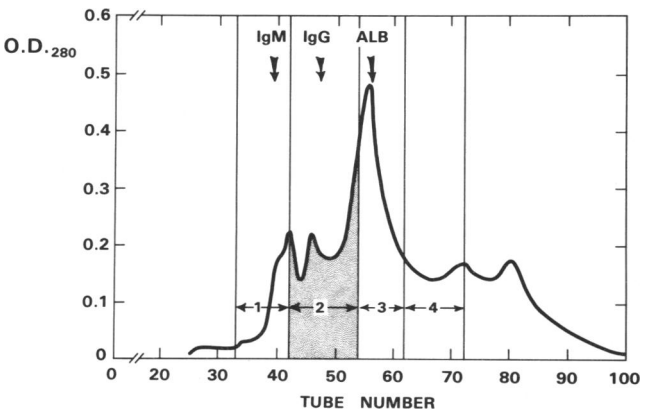

Fig. 2. Gel filtration of culture filtrate from fermenter-grown *A. fumigatus* (strain 507) on AcA 44 in PBS. Column was 2·5 × 100 cm and 150-drop fractions (*ca* 6·5 ml) were collected in each fraction. The elution positions of human IgM, IgG and albumin are shown for reference. Pools of fractions were made as indicated by 1, 2, 3 and 4. The fractions in pool No. 2 (*shaded*) reacted with patients' sera by immunodiffusion.

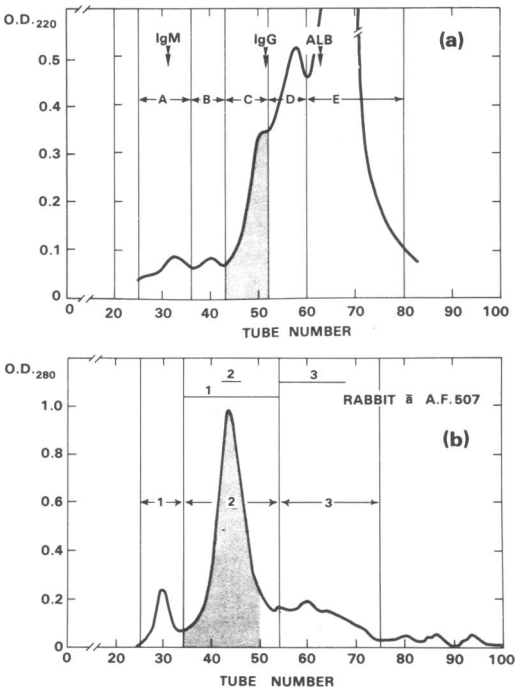

Fig. 3. (a) Gel filtration of culture filtrate, pool 2 (see Fig. 2) on Sephadex G-200 (2·5 × 100 cm) in PBS. Pools were made as indicated by A, B, C, D and E. Only fractions from pool C (*shaded*) reacted with patients' sera. Elution positions of human IgM, IgG and albumin are shown for reference. (b) Gel filtration of cell sap extract from mycelia of fermenter-grown *A. fumigatus* (strain 507) on G-200 (as in *a*). Pools of fractions made as indicated by 1, 2 and 3. The major reaction with patients' sera was with pool 2 (*shaded*), but an additional reaction was also exhibited by pool 3. Pool 2, but not pool 3, also reacted with rabbit antiserum made against *A. fumigatus* CF.

reactions are usually not very strong, but can be sufficiently intense to show relationsips. A comparison of stationary CF from three strains of *A. fumigatus* (strains 507, 515 and 534) shows some common and some strain-specific antigens with respect to individual sera.

Fractionation of fermenter CF and CS. Gel filtration of fermenter CF on AcA 44 is shown in Fig. 2. Tubes showing reactions with sera from aspergilloma patients are indicated in the shaded area (pool 2) of the elution curve. This area corresponds to the elution volume of human IgG. The reactive fractions were pooled and refractionated on Sephadex G-200 as shown in Fig. 3a. The fractions exhibiting reactions with patients' sera again eluted in the same volume as IgG, but the heterogeneity of the CF was still evident. In contrast, fermenter-grown CS shows a relatively simple elution curve on G-200 (Fig. 3b) with the major peak showing the reaction with patients' sera. In addition, this peak reacted with rabbit antiserum made against *A. fumigatus* CF from stationary cultures. The rabbit antiserum also reacted with fractions comprising pool 3. No reactions were observed with pool 1 fractions with either patient or rabbit sera. The shaded area (pool 2) which reacts with patients' sera was found to elute slightly ahead of the positive fractions from the CF, suggesting that the reactive components in the CS were slightly larger than the reactive components of the CF. The purified CF components are referred to as CF2C and the purified CS components are referred to as CS2.

Characterization of CF2C. CF2C was found to contain 12·5% neutral hexose. On SDS gel electrophoresis, CF2C was found to be a single component with a molecular weight of 123,000 daltons. In the presence of reducing agents, the molecular weight fell to 33,000 daltons. A second minor component can also be detected after reduction with a molecular weight of approximately 70,000 daltons (Fig. 4). The major component of the AcA fraction 2 appears to be contained in

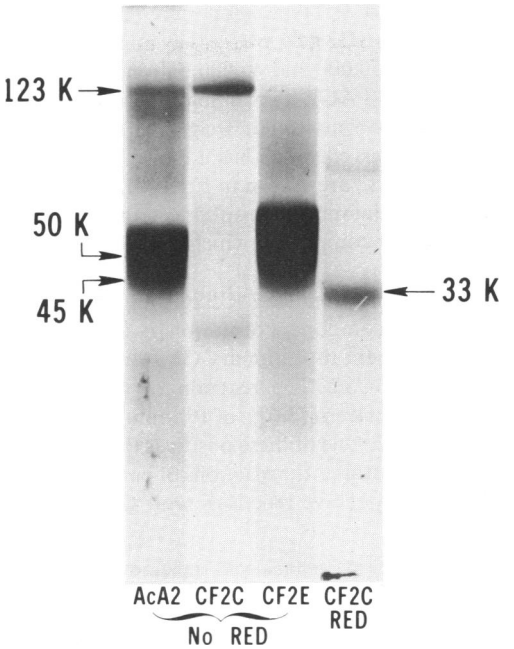

Fig. 4. SDS-PAGE on 7·5% polyacrylamide gels (no red, no reduction; red, after reduction) of purified CF antigen from *A. fumigatus* (strain 507) fermenter-grown. AcA2 = pool 2 from AcA 44 fractionation; CF2C = pool C from G-200 fractionation; CF2E = pool E from G-200 fractionation; CF2C RED = after reduction of CF2C. Pool AcA2 has two closely migrating components (50 and 45K) in addition to the 123K component. Anode is at the bottom of gel.

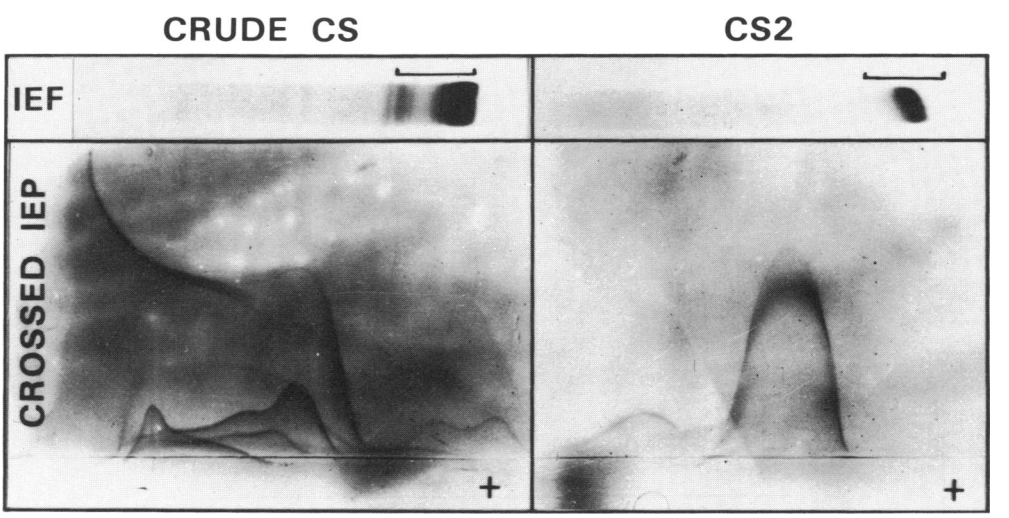

Fig. 5. Isoelectric focusing (IEF) of mycelial cell sap from fermenter-grown *A. fumigatus* (strains 507 and CS2) (Fig. 3b) on polyacrylamide gel (pH gradient 3·5–9·5). Protein was fixed with 12% TCA and stained with Coomassie brilliant blue. Crossed-immunoelectrophoresis (IEP) was performed by running the electrophoresed proteins at right-angles into 1% agarose containing 10% rabbit antiserum made against CF at pH 8·6 (0·05 M barbital buffer) for 16 hr at 1–2 V/cm. Plates were fixed and stained.

CF2E and elutes from G-200 after CF2C. This component (CF2E) does not appear to react with patients' sera by double diffusion. On SDS gels, two closely migrating components can be discerned in CF2E with molecular weights of 50,000 and 45,000 daltons.

Characterization of CS2. On SDS-PAGE, two major components are observed in CS which also elute in CS2. These components have molecular weights of 182,000 and 38,000 daltons. After reduction a single component is observed which has a molecular weight of 45,000 daltons.

Isoelectric focusing of CS and CS2 are shown in Fig. 5. The single component of CS2 has an isoelectric point of 5·2–5·6. Crossed immunoelectrophoresis against rabbit anti-*A. fumigatus* strain 507 shows a major peak and a minor component which exhibits partial immunological identity of the major peak (Fig. 5).

Immunological relationship of CF2C and CS2. CF2C and CS2 appear to be immunologically identical to each other as well as to a component of stationary CF by double immunodiffusion. The same antigen also appears to be present in stationary CF from *A. fumigatus* strains 515 and 534.

Reaction of patients' sera with CS2. The reaction of CS2 with sera from patients with aspergilloma is shown in Table 1. Fourteen (73·6%) of 19 confirmed cases showed positive reactions of varying intensity with isolated CS2. One of these positives (No. 6) was very weak but was absent in unfractionated CF. Table 2 demonstrates the reactions of patients with aspergillosis. Ten (76·9%) of 13 patients with ABPA showed positive reactions with CS2. One of the negatives was also

Table 1. Analysis of sera of patients with aspergilloma with standard CF and purified CS antigens

	Reaction of serum with *Aspergillus fumigatus* (strain 507)	
Patient No.	Standard CF*	CS2†
Aspergilloma		
1	+	++
2	+	++
3	+	++
4	+	−
5	+	++
6	−	±
7	+	−
8	+	++
9	+	+++
10	+	++
11	+	+++
12	+	−
13	+	−
14	+	++
15	+	++
16	+	+
17	+	−
18	+	++
19	+	++
20‡	+	++
21‡	+	−
22‡	+	+
23‡	+	+

* Used at 10 mg/ml concentration (dry weight). Stationary cultures.

† 1·8 optical density units at 280 nm (approximately 0·9 mg/ml) used as concentration.

‡ Unconfirmed.

Table 2. Precipitin analysis of sera of patients with aspergillosis with standard CF and purified CS antigens

Patient No.	Reaction of serum with *Aspergillus fumigatus* (strain 507)	
	Standard CF*	CS2†
ABPA		
24	+	++
25	+	+++
26	−	−
27	+	±
28	+	−
29	+	+
30	+	++
31	+	+
32	+	+
33	+	±
34	+	+++
35	+	−
36	+	+
37‡	+	±
Farmer's lung disease		
38	+	+
39	−	−
40	−	−
Other fungal disease		
6 patients	−	−
Normal human sera		
5 subjects	−	−

* Used at 10 mg/ml concentration (dry weight). Stationary cultures.

† 1·8 optical density units at 280 nm (approximately 0·9 mg/ml) used as concentration.

‡ Invasive.

negative with 507 standard CF (No. 26), and one patient with invasive aspergillosis also reacted with CS2 (No. 37). Six patients with other fungal diseases and five normal human sera were all negative with both standard CF and CS2.

DISCUSSION

Pepys (1978) has pointed out the importance of precipitating antibody in the pathogenesis and diagnosis of Aspergillus-related disease. Atopic individuals with precipitating antibody to *A. fumigatus* extracts are likely to develop ABPA, while precipitins may also be found in sera from non-atopic individuals with extrinsic allergic alveolitis, aspergilloma or invasive aspergillosis. Precipitin reactions are usually strong and multiple with aspergilloma patients and weaker in ABPA patients (Kurup & Fink, 1978). With the latter it is important to use a battery of extracts to ensure a positive reaction (Longbottom & Pepys, 1964).

Previous studies from this laboratory have demonstrated that CF obtained from stationary cultures of 11 strains of *A. fumigatus* may be used to detect precipitating antibody in sera of Aspergillus-related diseases with varying degrees of success (Kurup *et al.*, 1978). Three strains were selected to be used for general testing of patients (Kurup *et al.*, 1980). One strain, 507, shows strong reactions with 29 of 33 Aspergillus-related disease sera (Kurup *et al.*, 1978). The purpose of this

study was to isolate and characterize antigens from this strain in order to facilitate the standardization of these antigens. To accomplish this we had to change from stationary cultures to fermenter-grown cultures so that large quantities of protein for isolation procedures could be prepared. In so doing our results indicate that little if any loss occurred in the antigenic quality of the CF produced.

A major antigenic component was purified from the CF of fermenter-grown cultures of *A. fumigatus* (strain 507). An immunologically identical component (CS2) was purified from the cell sap. CS2, however, chromatographed at a slightly lower elution volume than CF2C, indicating a somewhat larger molecular weight. The elution volume of these antigens corresponded to molecular weights of approximately 150,000 to 200,000 daltons.

On SDS gels, CF2C appears to have a molecular weight of 112,000 daltons without reduction and 33,000 daltons after reduction. An additional, very minor component is seen after reduction with a molecular weight of approximately 70,000 daltons. Whether this minor component represents an intermediate between the unreduced and reduced state is not clear at present. In the absence of a reducing agent, CS2 shows two bands of SDS gel electrophoresis. One of the components has a molecular weight of 166,000 which is consistent with a CS component related to CF2C with a higher molecular weight. The nature of the 39,000 molecular weight band, also found on this gel, is not certain. Whether or not it is related to the higher molecular weight component or is a contaminant is also unclear. It appears to be different from the reduced form of CS2 which exhibits a molecular weight of 45,000 daltons. No other band was observed after reduction of CS2.

These results are best interpreted as a tetrameric structure for both CF2C and CS2. The basic subunits of these antigens differ by 10,000 to 12,000 daltons which accounts for the difference in molecular weights. At present, we do not know if all four subunits are identical or just similar in molecular weight. In addition, it is not clear if the molecular weight change occurs during secretion from the cell or is an artifact occurring during isolation procedures. Some support for the latter arises from the CRIEP of CS2 which shows a major component with an isoelectric point of 5·2–5·6 and an antigenically related minor component at a slightly more basic position. This minor component may represent a smaller degradation product.

When tested against patients' sera, CS2 was found to be an important antigenic component of the 507 standard CF since it reacted with approximately 75% of the sera from patients with aspergilloma and aspergillosis which react with the standard antigen. In addition, it appears to be a component of strains 515 and 534 as well. Work is now in progress to examine the component of *A. fumigatus* CF which reacts with the sera of patients which do not precipitate CS2.

Kim *et al.* (1978) and Kim & Chaparas (1978) recently reported on their attempts to prepare purified antigens from *A. fumigatus* mycelia after a 4-day growth on synthetic media. By using fused-rocket immunoelectrophoresis with antisera raised in rabbits, they could discern 40 precipitin reactions. On disc electrophoresis they were able to differentiate 35 bands staining with Coomassie blue. The large number of components obtained on gel electrophoresis by these investigators is surprising. While SDS gel electrophoresis of our CS revealed a very heterogeneous pattern, disc electrophoresis of the same sample exhibited a very simple pattern.

Kim *et al.* (1978) subjected their mycelial extracts to gel filtration on Sephadex G-75 (70,000 dalton exclusion limit for proteins) and obtained a fraction in the excluded volume which exhibited 17 bands on disc electrophoresis and 21 fused-rocket precipitins. This fraction was also very active in skin tests and lymphocyte transformations using sensitized guinea-pigs. More recently, they have examined this fraction and several other extracts with sera from patients with aspergillosis and aspergilloma (Kim & Chaparas, 1979; Kim, Chaparas & Buckley, 1979; Chaparas *et al.*, 1980). The void volume fraction was found to give positive reactions by immunodiffusion with most sera tested from patients with ABPA (67%) and aspergilloma (92%). These investigators concluded that no preparation was able to detect all positive cases of Aspergillus-related disease. In addition, the variations in quality and quantity of antigens from each strain, as well as the lot-to-lot variations, can be expected to contribute to unreliability of antigen preparations. Only by the isolation of defined antigens which can be mixed in defined concentrations can such antigen reliability be approached. We have attempted to prepare and characterize at least one component which will be useful for such a reagent.

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