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Antigens of *Aspergillus fumigatus*

II. ELECTROPHORETIC AND CLINICAL STUDIES

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Summary. Cell sap (CS) and culture filtrate (CF) preparations of *Aspergillus fumigatus* strains Ag-507, Ag-515, and Ag-534 were analysed by two dimensional electrophoresis (2-DE; i.e., first dimension isoelectric focusing, second dimension sodium dodecyl sulphate gradient pore gel), which enabled detection of strain- and species-specific components. In CS preparations it was shown that CS2, a fraction isolated from strain Ag-507 by gel filtration, consists of the major protein components in the CS of the three *A. fumigatus* strains tested. Culture filtrate preparations of the three *A. fumigatus* strains analysed by 2-DE exhibited patterns dissimilar to the CS patterns, as well as to each other, presumably due to proteolysis. Culture filtrate preparations are therefore a less reliable source of standardized antigens than CS preparations. CS2 has a major component with a mol. wt. of approximately 150,000 and an s_{app} of 6·3 S. CS2 reacts on immunoelectrophoresis, producing one major precipitin arc with aspergilloma or allergic bronchopulmonary aspergillosis (ABPA) patient sera. Antibody titres of the IgG and IgA classes to CS2, as measured by enzyme-linked immunosorbent assay (ELISA), were demonstrated to be similar in aspergilloma and ABPA patients; IgG titres were higher than IgA. Similar titres were also obtained utilizing sera of patients that did or did not exhibit precipitating antibodies to CS2. In the diagnosis of ABPA, skin tests with CS2 were comparable in specificity to currently available commercial preparations. Importantly, CS2 is a standardized major antigenic preparation of the CS of three *A. fumigatus* strains which has been shown to be diagnostically useful.

Abbreviations: 2-DE, two-dimensional electrophoresis; CF, culture filtrate preparation; CS, cell sap preparation; ELISA, enzyme-linked immunosorbent assay; IEP, immunoelectrophoresis; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; TEMED, *N,N,N',N'*-tetramethylethylenediamine; Tween, Tween-20; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulphonyl fluoride; pI, isoelectric point.

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INTRODUCTION

Previously, Calvanico *et al.* (1981) reported the preparation and partial characterization of mycelial extracts and culture filtrates (CF) of *Aspergillus fumigatus*. An antigen was partially purified from the cell sap (CS) of *A. fumigatus* strain Ag-507 and designated CS2. It exhibited precipitin reactions with 75% of the sera from patients with aspergilloma and allergic bronchopulmonary aspergillosis (ABPA).

Many species of *Aspergillus* can produce disease (Mahgoub & Hassan, 1972; Gerber & Jones 1973; Kim & Chaparas, 1979), and the identification of antigenic components is useful in the diagnosis of aspergillosis. The standardization of these antigens would be extremely helpful in establishing reliable criteria for this disease. Three strains of *A. fumigatus* have been used to prepare antigenic extracts which react with most patient sera (Kurup *et al.*, 1978). In order to pursue our studies on the characterization of CS2 and to explore other potentially useful components of the CS and CF, we have employed two-dimensional electrophoresis to characterize the protein components of these extracts (Anderson & Anderson, 1977). In addition, we have evaluated the clinical usefulness of CS2.

MATERIALS AND METHODS

Organisms and culture conditions

Three strains of *A. fumigatus*, namely Ag-507, Ag-515 and Ag-534, were used in this study. Characterization of these strains was previously reported by Kurup *et al.* (1978). Strain Ag-507 was isolated from the sputum of a patient with aspergilloma, while Ag-515 was received from Northern Regional Research Laboratories (Peoria, IL) and Ag-534 from the Allergy Department, University of Wisconsin-Madison. As previously described (Calvanico *et al.*, 1981), cultures were grown in a synthetic broth in a fermenter.

Culture filtrate material

Culture filtrate material was prepared for each of the three *A. fumigatus* strains as previously reported (Calvanico *et al.*, 1981). The CF of each of the three *A. fumigatus* strains were concentrated to approximately 25 mg/ml and stored at $-70°$.

Cell sap material

The mycelial phase of the 4-day fermenter growth of each of the three *A. fumigatus* strains were ground with glass beads to release CS as previously reported (Calvanico *et al.*, 1981). The CS preparations were stored at $-70°$ and protein content of the CF and CS preparations were approximated by spectrophotometric readings at 280 nm using $E^{1\%}_{1cm} = 10{\cdot}0$.

Gel filtration

Gel filtration was performed on a Sephadex G-200 column ($100 \times 2{\cdot}5$ cm) equilibrated with phosphate-buffered saline (PBS; pH 7·2; 0·1 M). Elution was carried out with the same buffer.

Immunodiffusion

Double diffusion was performed by modification of the techniques of Ouchterlony (1975). The gels were stained with 1% Amido black in methanol-distilled water–acetic acid (4·5:4·5:1) after washing and drying.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Following the methods of Weber, Pringle & Osborn (1972), SDS-PAGE was performed on 7·5% gels. Gels were fixed overnight in 50% methanol/12% acetic acid in distilled water and stained with Coomassie blue G-250.

Immunoelectrophoresis

Electrophoresis of protein samples was performed for 80 min at 4 mA/cm on microscope slides as described by Grabar (1959). The developed IEP plates were washed for 24 hr in 0·01 M borate buffer (pH 7·0) followed by a water wash for 24 hr. The IEP plates were air-dried and stained with 1% Amido black in 7% acetic acid and destained in 7% acetic acid.

Analytical ultracentrifugation

Analytical ultracentrifugation was performed on the Spinco Model E analytical ultracentrifuge (Beckman, Palo Alto, CA). The sample was placed in a standard double-sector cell with solvent being utilized for the reference. Centrifugation was performed at 52,600 r.p.m. Photographs of the schlieren patterns were taken at 8-min intervals and the apparent sedimentation coefficient (s_{app}) was determined (Chervenka, 1973).

Two-dimensional electrophoresis

Two-dimensional electrophoresis (2-DE) was performed by the techniques of O'Farrell (1975). Isoelectric focusing was performed with 130×3 mm ID glass tubes. The cathode buffer was 10 mM histidine and the anode buffer was 10 mM phosphoric acid. Samples for isoelectric focusing were either not reduced or reduced in sample treatment buffer (0·0625 M tris-HCl, pH 6·9, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol) for 7 min at 22°. After focusing, gels loaded with 100 μg protein were fixed for staining in 45% methanol/12% acetic in water for 24 hr and then stained with Coomassie blue R250 (27% isopropanol, 10% acetic

acid, 0·04% Coomassie blue R250, 0·5% $CuSO_4$ in water) for 1½ hr, and destained in 7% acetic acid/5% methanol in water. Gels loaded with 300 μg protein (reduced) were placed in treatment buffer for 1 hr prior to electrophoresis in the second dimension. The second-dimension electrophoresis was performed utilizing 1·5 mm thick polyacrylamide slabs (16·5 × 7·4 cm) which were cast as an exponential gradient (10–20%) of polyacrylamide separating gel, utilizing the Bio-Rad 600 vertical slab gel unit and Bio-Rad 230 gradient former (Bio-Rad Laboratories, Richmond, CA). Three-microlitre volumes of low molecular weight standards (Pharmacia, Piscataway, NJ) were applied on the right-hand side of each slab. Electrophoresis was performed at 30 mA/slab until the tracking dye front reached the bottom of the slab gel. The gels were fixed in 5% methanol/12% acetic acid in water for 24 hr and stained with Coomassie blue (0·125% R250, 50% methanol, 10% acetic acid in water) for 24 hr. The stained slabs were destained in 50% methanol/12% acetic acid in water and then dried on a slab gel dryer (Model S280; Hoefer Scientific Instruments, San Francisco, CA). Alternatively, the fixed polyacrylamide slabs were washed for an additional hour in 10% ethanol/5% acetic acid in water and stained with silver as described by Merril (1981). The silver-stained gels were then washed for several days in 1% acetic acid and dried.

Antiserum

Antiserum to *A. fumigatus* strain 507 CS was raised in New Zealand white rabbits by multiple subcutaneous injection of CS in Freund's complete adjuvant.

Human sera

Sera from patients with aspergilloma, ABPA or other fungal diseases were donated by Dr Jordan N. Fink (Department of Medicine, the Medical College of Wisconsin, Milwaukee, WI) and Dr Roy Patterson (Department of Medicine, Northwestern School of Medicine, Chicago, IL) and stored frozen at −70°.

Enzyme-linked immunosorbent assay (ELISA) for specific antibodies to CS2 of A. fumigatus *strain Ag-507*

A modification of the technique of Voller, Bidwell & Bartlett (1976) was utilized. Disposable cuvettes (Cuvettepack, Gilford Instruments Inc., Oberlin, OH) were utilized as a carrier surface for the antigen. The antigen utilized in ELISA was CS2 of *A. fumigatus* strain Ag-507 isolated by gel filtration on G200, as previously described (Calvanico *et al.*, 1981). The concentration of CS2 (which gave optimal binding) was 50 μg/ml in carbonate buffer (pH 9·6). A 200-μl volume of diluted antigen was utilized to coat the cuvettes at 4° overnight. The cuvettes were emptied and washed three times with PBS-Tween.

The sera of patients were diluted 1:5000 in PBS-Tween, for IgG assay and 1:1000 for IgA assay. Rabbit anti-human class specific (anti-IgG or anti-IgA) sera were diluted to 1:3000 in PBS-Tween. Alkaline phosphatase- labelled goat anti-rabbit IgG (prepared by conjugation with glutaraldehyde) was diluted to 1:3000. p-Nitrophenyl phosphate (Sigma 104 phosphatase substrate 1 mg/ml in diethanolamine buffer, pH 9·8) was used as substrate for alkaline phosphatase.

Dilutions of patient sera (200 μl) were then added to each cuvette. Eleven cuvettes were coated with antigen, one cuvette was utilized as a blank control (no antigen) and two received no serum. The cuvettes were incubated at room temperature for 3 hr, then washed three times with 500 μl of PBS-Tween. Two hundred-microlitre volumes of rabbit anti-human IgG or IgA were added to the appropriate cuvettes and incubated for 3 hr at room temperature and then washed three times with 500 μl of PBS-Tween. A 200-μl volume of diluted alkaline phosphatase-labelled goat anti-rabbit IgG was then added and the cuvettes were incubated for 3 hr at room temperature. After three washings with 500 μl of PBS-Tween, 200 μl volumes of substrate solution were added. The reaction was allowed to proceed at room temperature and stopped after 30 min by the addition of 5 μl of 4 N NaOH. Absorbance at 405 nm was determined utilizing a Gilford Manual EIA reader (Gilford Instruments Laboratories, Inc.). All serum samples were run in triplicate and the averages recorded. The average values of the titres of IgG or IgA for each patient with aspergilloma or ABPA were then subjected to a statistical *t* test, and hypotheses were tested at the 0·05 significance level. Titres of IgG or IgA between aspergilloma and ABPA patients were compared. Also, titres of IgG or IgA antibody of aspergilloma and ABPA patient sera that did or did not have precipitating antibodies to CS2 were compared.

Skin tests

Skin prick tests were performed and read according to standard methods (Sheldon, Lowell & Mathews, 1967) to assay for immediate hypersensitivity to CS2 and compared with results obtained from similar tests

with allergenic extracts of *A. fumigatus* obtained commercially (Holister-Stier, Spokane, WA, lot No. L10911504; Greer Laboratories, Lenoir, NC, lot No. LM 3-26; Center Laboratories, Port Washington, NY, lot No. 8021169; and Allergy Laboratories Inc., Oklahoma City, OK, lot No. 2798). The skin tests were performed with the informed consent of patients seen and evaluated in the Allergy Clinic of the Medical College of Wisconsin at the Milwaukee County Medical Complex. The concentrations of all preparations utilized in the skin tests was approximately 0·60 mg/ml. Results (wheal and flare) were evaluated after 15 min.

RESULTS

Two-dimensional electrophoresis

The 2-DE patterns of the CS proteins of the three strains of *A. fumigatus* are shown in Fig. 1 (I-IV). As many as 150 spots were observed in the CS electrophoretograms. The majority of the basic components had molecular weights ranging from 30,000 to 94,000. The high resolution of 2-DE enabled detection of strain- and species-specific components, as indicated on the composite diagram of the CS preparations of the three strains (Fig. 2). Approximately 80 spots were found to be common to all three strains of *A. fumigatus*. In addition, there were spots which were specific for each of the three strains. There were also spots which were common to strains Ag-515 and Ag-534 but not Ag-507. Highly sensitive silver staining (limit of detection of *ca* 0·05 ng/mm^2) was used to verify the absence of suspect strain-specific components of the composite CS electrophoretogram (Fig. 2). For example, the silver stained electrophoretogram in Fig. 1 (I-IV) confirms the absence of specific spots in

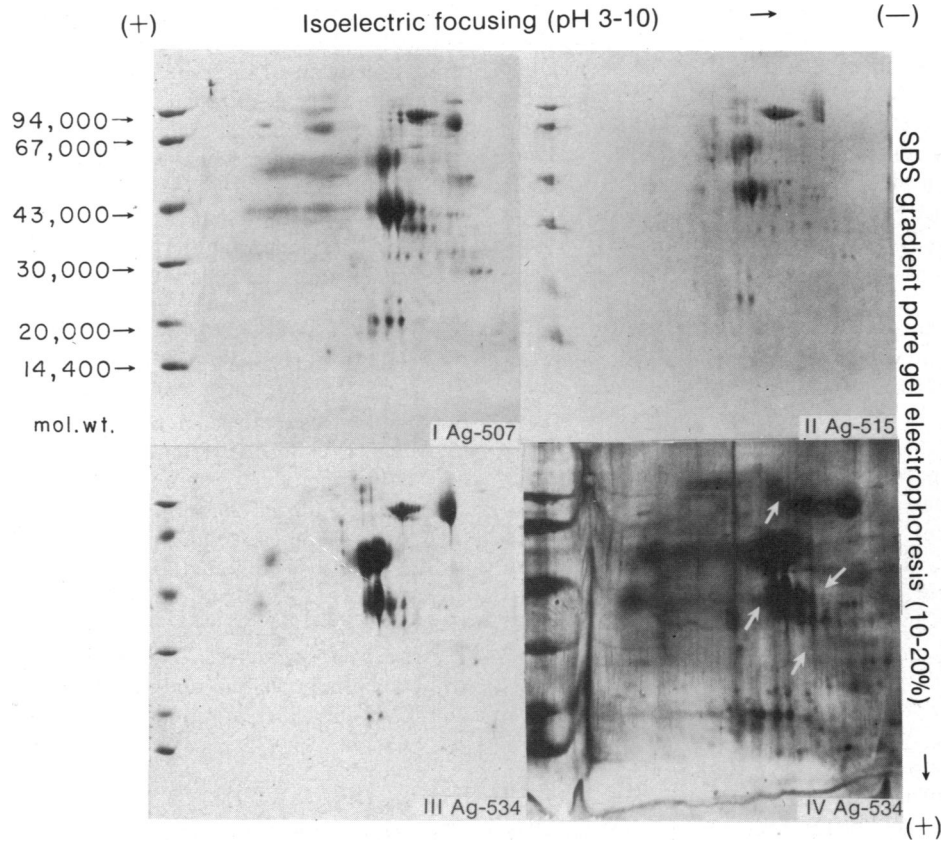

Figure 1. CS components of three *Aspergillus fumigatus* strains (Ag-507, Ag-515, Ag-534) as indicated, separated by 2-DE. I–III, stained with Coomassie brilliant blue R-250; IV, stained with silver. *Arrows* on IV indicate missing CS components present in the electrophoretograms of strains Ag-507 and Ag-515.

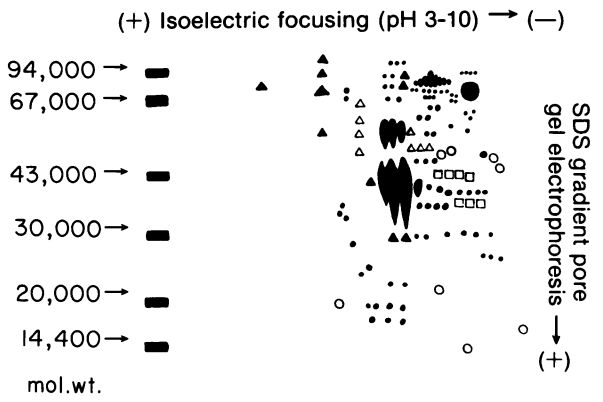

Figure 2. Composite diagram of the CS components. 2-DE patterns of three *A. fumigatus* strains (Ag-507, Ag-515, Ag-534) demonstrating strain- and species-specific components. (●) Components common to all three strains; (▲) components specific to strain Ag-507; (□) components specific to strain Ag-515; (○) components specific to strain Ag-534; (△) components common to strains Ag-515 and Ag-534.

strain Ag-534 which are present in the CS electrophoretograms of strains Ag-507 and Ag-515 stained with the less sensitive Coomassie blue. In general, however, silver staining did not reveal anything which was undetectable by Coomassie blue staining. Differences between the CS preparations of the three strains were also detected by immunodiffusion. When the CS of the three strains of *A. fumigatus* were analysed by immunodiffusion with rabbit antiserum to Ag-507 CS, strains Ag-515 and Ag-534 showed reactions of partial identity with strain Ag-507. Five batches of each strain were examined by each technique with similar results.

The 2-DE patterns of the CF preparations of the three strains of *A. fumigatus* are shown in Fig. 3. The patterns were different from those exhibited by the CS preparations. As many as 200 spots were visible and, in addition, by contrast to the CS preparations, many CF spots were more acidic and had lower molecular weights (14,000 to 30,000). The CF electrophoretograms also demonstrated considerable smearing. The 2-DE patterns of strains Ag-507 and Ag-515 appeared to be similar, whereas the pattern of strain Ag-534 appeared to be less related to the others. The three CF preparations showed significantly less similarity to each other than the three CS preparations.

Characterization of CS2 from strain Ag-507

CS2 prepared by gel filtration on G200 (Calvanico *et al.*, 1981) was characterized by 2-DE. The pattern obtained is shown in Fig. 4. Three major components corresponding to the major components of whole CS were observed. Two of the components have similar isoelectric points, while the heavier component is slightly more basic.

On SDS gels (Fig. 5), the molecular weight corresponding to the heavier component was 81,000, while that of the two components with similar isoelectric points were 41,000 and 53,000. Non-reduced CS2 produced two bands with molecular weights greater than 200,000, a major band with a molecular weight of 120,000 to 150,000 and two minor bands with molecular weights of 38,000 and 58,000.

In the analytical ultracentrifuge, CS2 sedimented as a major peak with an s_{app} of 6·3 S. Smaller amounts (10–15%) of higher and lower molecular weight components were also observed.

IEP of CS2 and CS preparations of *A. fumigatus* strain Ag-507, utilizing serum from a patient with aspergilloma, reacted with CS2 to produce one major precipitin arc on IEP, while the same patient's serum reacted with whole CS to produce at least three precipitin arcs. The same reactions were observed utilizing sera from two additional aspergilloma patients. Sera from two ABPA patients also exhibited one major precipitin arc on IEP with CS which appeared to be identical to aspergilloma patients, and at least three precipitin arcs with whole CS. Sera from four patients with various other fungal diseases did not show any reactions with CS2 or CS. Rabbit antisera raised to *A. fumigatus* strain Ag-507 gave a single precipitin reaction with CS2 and at least three precipitin reactions with whole CS.

The results of ELISA for antibody to CS2 are shown

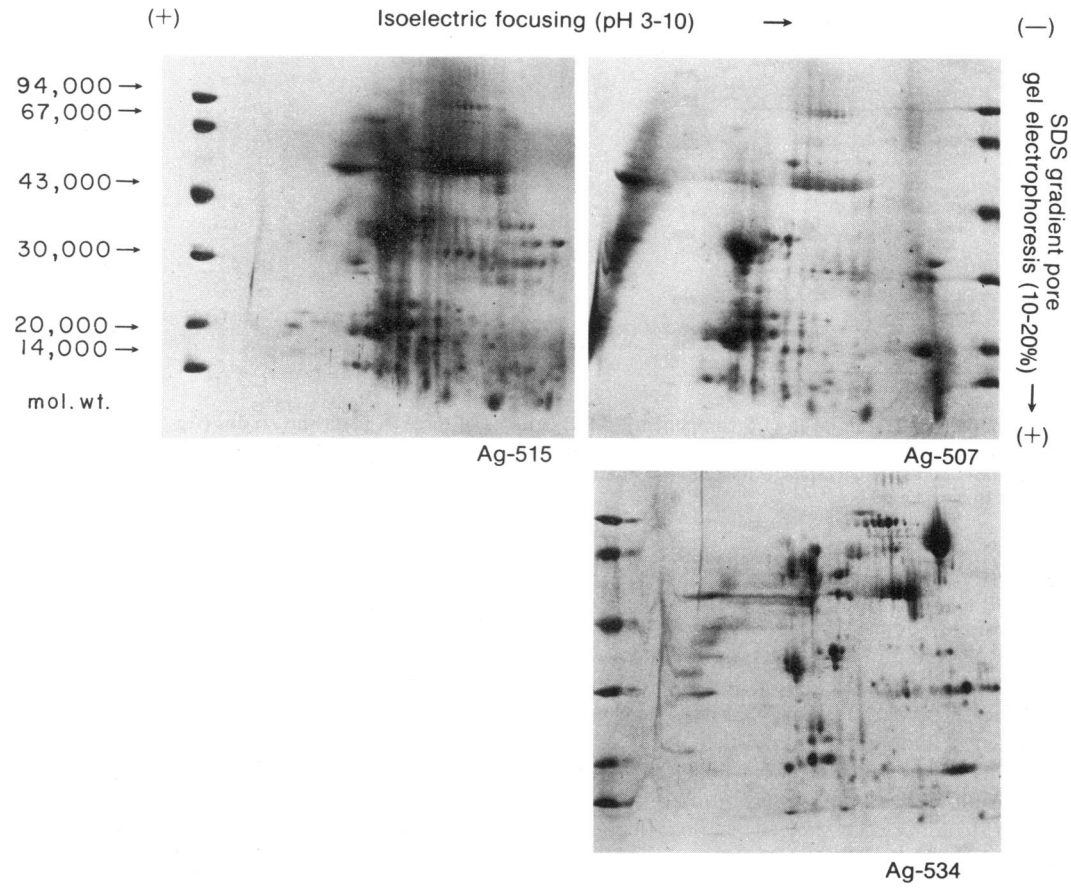

Figure 3. CF components of three *A. fumigatus* strains (Ag-507, Ag-515, and Ag-534) as indicated, separated by 2-DE, strained with Coomassie brilliant blue R-250.

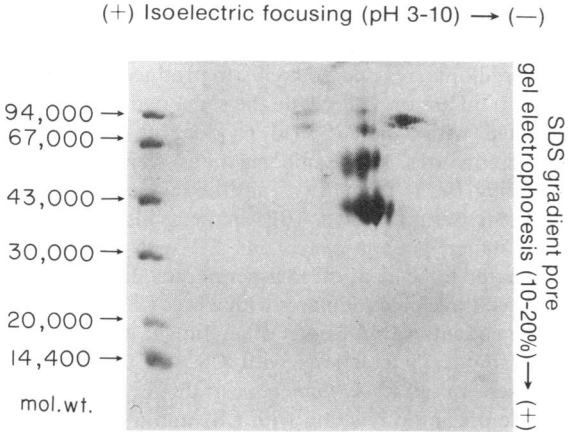

Figure 4. CS2 components separated by 2-DE and stained with Coomassie brilliant blue R-250.

in Fig. 6. Patients with precipitating antibody were compared to patients without precipitating antibody utilizing a *t* test. When tested at the 0·05 significance level, there was no statistical difference in titres of IgG (or IgA) of aspergilloma or ABPA patient sera which did or did not have precipitating antibodies to CS2. In addition, IgA titres generally correlated with IgG titres in each individual, i.e. where IgG was high so was IgA, and where IgG was low so was IgA. The IgG (and IgA) titres of both aspergilloma and ABPA patients appeared similar, although those of the former group tended to reach higher levels in certain individuals. When tested by a *t* test at the 0·05 significance level, however, there was no significant statistical difference in IgG (or IgA) titres in either disease group.

The results of patient skin tests utilizing CS2 are presented in Table 1. CS2 evoked an immediate

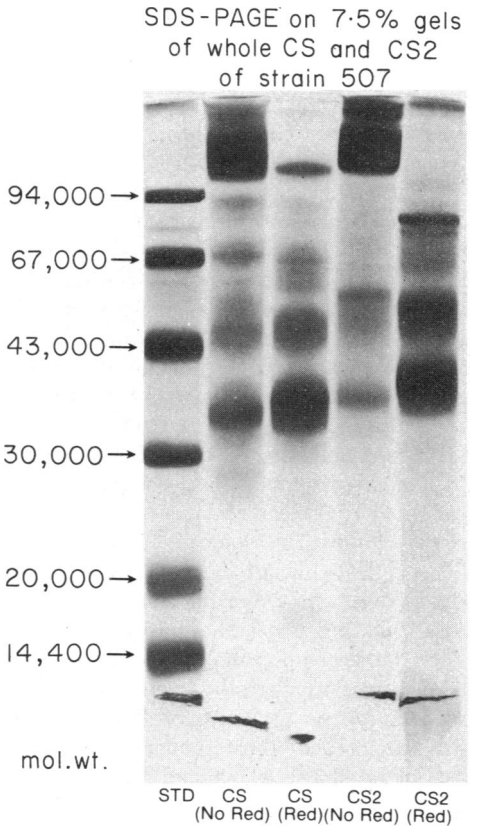

Figure 5. SDS-PAGE on 7·5% gels (no red, no reduction; red, after reduction; STD, standards) of whole CS and CS2 of *A. fumigatus* strain Ag-507. Anode is at the bottom.

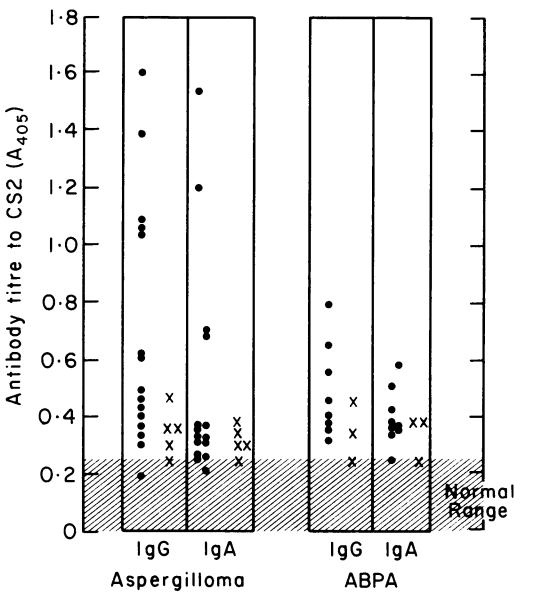

Figure 6. Enzyme-linked immunoabsorbent assay (ELISA) data utilizing CS2 as antigen, and aspergilloma and ABPA patients' sera. (●) Sera showing precipitating antibody to CS2; (×) sera showing no precipitating antibody to CS2. Titres determined spectrophotometrically at 405 nm.

Table 1. Result of skin tests performed on patients with the indicated allergic disease utilizing commercially prepared antigens from *A. fumigatus* from the indicated sources and CS2 from this study

Source	Asthma	ABPA
Holister-Stier Laboratories	8/24	3/4
Greer Laboratories	6/24	4/4
Center Laboratories	3/24	2/4
Allergy Laboratories of Oklahoma	3/24	3/4
CS2	6/24	2/4

No. of patients responding positively/No. of patients tested.

hypersensitive skin response in two out of four ABPA patients tested and in six out of 24 asthma patients. Results obtained with commercial preparations of *A. fumigatus* are also presented in Table 1. The data indicates that CS2 is somewhat less specific for ABPA patients than other commercially available preparations, i.e. positive skin test with 50% of the ABPA patients tested and 25% with atopic individuals without ABPA. The best results were obtained with Greer Laboratories (100% with ABPA, 25% with atopics) and Allergy Laboratories of Oklahoma (75% with ABPA, 12·5% with atopics).

DISCUSSION

Considerable variability in the composition of commercial preparations of *A. fumigatus*, utilized to test patients with *Aspergillus*-related diseases, exists. Kim & Chaparas (1979) evaluated commercial and experimental preparations of *A. fumigatus* by serological methods. Major qualitative differences were detected in commercial antigen preparations of *A. fumigatus* by crossed-immunoelectrophoresis, although all commercial preparations reacted with patient sera. Fink *et al.* (1977) pointed out the wide variability in reactivity of patients sera due to the relatively crude nature of antigen preparations; however, Kurup & Fink (1978)

demonstrated the diagnostic importance of precipitin reactions for aspergilloma and ABPA patients.

As previously reported from this laboratory, three strains of *A. fumigatus* were utilized for general testing of patients with *Aspergillus*-related disease (Kurup *et al.*, 1979). Since strain Ag-507 showed strong reactions with 29 of 33 patient sera with *Aspergillus*-related disease, it was selected as a source of antigens for isolation and characterization. Cell sap was fractionated and the major fraction was designated CS2. The purpose of the present study was to compare CS and CF protein components of the three major antigenic strains of *A. fumigatus* (Ag-507, Ag-515, Ag-534) by high resolution techniques. Additionally, CS2 was further characterized by high resolution techniques and also utilized in IEP, and particularly ELISA and skin tests to evaluate its clinical usefulness.

When the CS of the three strains of *A. fumigatus* were subjected to 2-DE, a high degree of similarity of the CS preparations was observed. As many as 150 spots were present in the CS preparations. The high resolution of 2-DE enabled the detection of strain- and species-specific components. These patterns were reproducible with several preparations of CS. Supportive evidence for strain differences, based on the proteins of the CS of the three *A. fumigatus* strains resulted from immunological comparison by double diffusion. When CF preparations of each of the three *A. fumigatus* strains were analysed by 2-DE, patterns different from those of the CS preparations were observed. In addition, they showed significantly less similarity between strains than the CS preparations. The lack of similarity of the CF electrophoretograms may be due to proteolysis, since many spots were detected in the lower molecular weight range. The CS preparations therefore appear to be a more reliable source of antigens which may also be related to their being more conservative proteins (Dickerson, 1972).

Two-dimensional electrophoresis demonstrated that CS2 consists of the major components of the CS protein of all three strains examined. This explains the high degree of cross-reactivity of the CS preparations with various sera. The major component of CS2 has a molecular weight of 150,000–180,000 by gel filtration. On SDS-PAGE without reduction, the major component had a molecular weight of at least 120,000. A more precise evaluation could not be obtained since the migration was only into the top portion of the gel which is the least reliable area for molecular weight determinations. Two minor components with molecular weights greater than 200,000 and two minor components with molecular weights of 38,000 and 58,000 were also detected on SDS-PAGE. After reduction, three major bands with molecular weights of 41,000, 53,000 and 81,000 appeared. These molecular weights agreed well with the molecular weights of components observed in 2-DE of CS2. The basic component had a molecular weight of 81,000 and the two more acidic components had molecular weights of 41,000 and 53,000 and similar isoelectric points. When CS2 was sedimented in the analytical ultracentrifuge, a major peak with a calculated s_{app} 6·3 S appeared. This agrees with the 150–180,000 molecular weight indicated by gel filtration. Both high and low molecular weight protein components were also observed in the ultracentrifuge. The combined data may be interpreted as suggesting that the major component of CS2 consists of two disulphide-linked polypeptide chains, one of *ca* 80–85,000 molecular weight and the other 50–55,000 daltons. This would give a molecular weight of 130–140,000 to the unreduced protein which agrees reasonably well with the gel filtration and ultracentrifugation data. We suggest that the 41,000 component is a degradative component of the 53,000 component, since they have similar pIs. Alternatively, this CS2 component can be a protein of 170,000 molecular weight composed of three subunits with molecular weights of *ca* 40,000, 50,000 and 80,000. Additional studies are needed to clarify the subunit structure.

As previously reported (Calvanico *et al.*, 1981), CS2 reacted on double diffusion with 75% of sera from patients with aspergilloma and aspergillosis. In this study, CS2 was utilized in immunoelectrophoresis, ELISA, and skin tests. When tested on immunoelectrophoresis, CS2 demonstrated one major precipitin arc with several aspergilloma and ABPA patient sera. Whole CS of strain Ag-507 demonstrated at least two additional minor precipitin arcs with the same aspergilloma patient sera. Immunoelectrophoretic analysis therefore demonstrated that CS2 consists of at least one major antigenic component with which aspergilloma and ABPA patient sera react. The additional reactions of patient sera with whole CS, indicate the presence of additional antigens in the whole CS preparations which are presently under investigation.

ELISA has been previously demonstrated as a useful technique for estimating titre of patients antibodies to an ammonium sulfate fraction of the culture filtrate of *Aspergillus* (Sepulveda, Longbottom & Pepys, 1979). CS2 antibody titres of IgG and IgA from ABPA and aspergilloma patients were determined by ELISA. IgG titres were higher than IgA titres in both

aspergilloma and ABPA patients. In addition, when statistically tested by the *t* test at the 0·05 significance level, there was no significant difference between titres of IgG or IgA in sera from patients exhibiting precipitins and those with no precipitins against CS2. The ELISA data indicated that CS2 is a useful diagnostic antigenic preparation particularly since patient sera that did not demonstrate precipitating antibodies to CS2 in double diffusion also reacted with CS2 in ELISA. These results indicate that some factor(s) other than titre is associated with precipitation of CS2 in gels, possibly the subclass distribution of antibody.

Skin tests have been established as useful diagnostic adjuncts for ABPA (Pepys, 1978). CS2 utilized in skin tests was demonstrated to be at least 50% as specific for ABPA as the best commercial preparations. It should be emphasized that CS2 represents a much more limited number of antigens than commercial preparations, and that its usefulness as a diagnostic antigen can be heightened by the addition of other standardized components, perhaps for example, CS2 prepared from additional *A. fumigatus* strains.

Reisman (1981) pointed out that the qualities of an ideal allergenic extract should include purity, potency, standardization and stability. In this study, we established some of these criteria for CS2. It was demonstrated to be clinically significant through ELISA and skin tests. More importantly, CS2 is a major antigenic fraction of the CS of *A. fumigatus* that has now been characterized.

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