

Immunologic significance of a collagen-derived culture filtrate containing proteolytic activity in *Aspergillus*-related diseases

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Background: Despite increasing evidence implicating fungal proteases in the virulence of pulmonary fungal diseases, routine fungal culture media do not favor protease production. Hence, filtrates that serve as the source of antigen for serologic determinations are poor in proteases, and consequently, the immunologic significance of these enzymes is unknown.

Methods: A clinical isolate of *Aspergillus fumigatus* was cultured on collagen medium, resulting in excretion of high levels of fungal proteases in the culture filtrate. This was compared with standard culture filtrates by diverse analytic techniques including immunoblotting with sera of patients with pulmonary aspergilloma (PA) and allergic bronchopulmonary aspergillosis (ABPA).

Results: Protein profiles of collagen medium filtrate showed several (glyco)proteins not found in conventional culture filtrates, including a prominent 32 kd glycoprotein, which coisolated in gel filtration and ion-exchange chromatography with elastase activity, as well as 67 kd and 94 kd (glyco)proteins. Intense IgG binding was seen with the 32 kd glycoprotein when ABPA and PA sera were used. The 94 kd protein showed intense binding with PA sera but not with ABPA sera, whereas for the 67 kd glycoprotein the reverse tended to be the case.

Conclusion: Fungal culture on collagen media results in the production of filtrates with high protease activity, containing unique (glyco)proteins of which at least one (32 kd) is closely associated with fungal elastase activity. These constituents are immunologically relevant, eliciting IgG production in patients with PA and ABPA, suggesting production of these (glyco)proteins during disease *in vivo*. The use of collagen media filtrates may enhance our serodiagnostic capacity in patients with fungal pulmonary diseases. (*J ALLERGY CLIN IMMUNOL* 1994;93:768-78.)

Key words: Protease, culture filtrates, *Aspergillus fumigatus*, allergic bronchopulmonary aspergillosis, aspergilloma, immunology, detection, IgG antibody, fungal elastase

Aspergillus fumigatus is an airborne pathogen, which predominantly affects the respiratory system. It is a cause of diverse respiratory disorders such as asthmatic reactions in atopic individuals (e.g., *Aspergillus* asthma and allergic bronchopulmonary aspergillosis [ABPA]), colonization of preexisting cavities leading to pulmonary as-

Abbreviations used

ABPA:	Allergic bronchopulmonary aspergillosis
CBB:	Coomassie Brilliant Blue
E-CCF:	Early-phase collagen culture filtrate
EDTA:	Ethylenediaminetetraacetic acid
E-SCF:	Early-phase Sabouraud culture filtrate
L-SCF:	Late-phase Sabouraud culture filtrate
PA:	Pulmonary aspergilloma
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
YCB:	Yeast carbon base

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pergilloma (PA), and additionally, invasive and disseminated mycosis in immunocompromised patients. In spite of its clinical importance, *A. fumigatus* accounts for only a small proportion of all airborne mold spores.¹ The virulence of *A. fumigatus* is due to specific properties by which it is able to bypass the cellular and/or humoral defenses of the host.²

Aspergillus species can produce extracellular proteases, and it has been suggested that such enzymes are involved in the pathogenicity of aspergillosis.³⁻⁵ Recently, attention has been focused on elastases as possible virulence factors of *Aspergillus*.⁶⁻¹⁰ Kothary et al.⁶ showed that strains of *A. fumigatus* isolated by air sampling were capable of producing an extracellular elastase and that the production of this enzyme was correlated with the ability to cause invasive aspergillosis in mice. Rhodes et al.⁷ examined 38 clinical specimens and reported that all isolates of *Aspergillus* species causing invasive aspergillosis produced elastase. *A. fumigatus* elastase was recently purified,^{3, 9} and enzymatic activity could be inhibited with monoclonal antibodies raised against the purified enzyme.¹¹

We investigated the immunologic significance of fungal proteases by culturing a clinical isolate of *A. fumigatus* in a culture medium promoting protease (e.g., elastase and collagenase) production. We¹²⁻¹⁴ and others¹⁵ have found that antigenic components are rapidly released in culture media that are useful in the detection of IgG and IgE antibodies in aspergillosis. An early-phase collagen culture filtrate (E-CCF) of *A. fumigatus* containing high levels of fungal elastase and collagenase activity was characterized and compared with early-phase and late-phase Sabouraud-glucose medium culture filtrates (E-SCF and L-SCF, respectively), which are routinely used as sources of antigen for serologic determinations. Protein profiles and immunologic characteristics (double immunodiffusion, IgG ELISA, and IgG immunoblotting patterns) were studied with the use of sera from patients with PA and ABPA.

METHODS

Sera

Sera from patients with proven PA ($n = 10$) or ABPA ($n = 10$) with elevated IgG ELISA levels and precipitating antibodies against *A. fumigatus* were used in the immunologic assays. Pooled sera were also prepared from these two patient groups. Sera from healthy individuals served as controls.

Cultures of *A. fumigatus*

The strain of *A. fumigatus* used for preparing the antigenic extracts was isolated from a patient with PA, as previously described.¹⁴ Spores harvested from *A. fumigatus* maintained on solid media containing Sabouraud agar were screened for their ability to grow on liquid medium containing elastin supplemented with yeast carbon base (YCB) (Difco Laboratories, Detroit, Mich.). One mycelial sphere growing on elastin-YCB medium was used to study conditions for optimal elastase and collagenase production in elastin- and collagen-containing media with various YCB concentrations. Borate buffer (0.2 mol/L, pH 8.0), which contained 0.2% insoluble collagen type I (from bovine Achilles tendon) (Sigma Chemical Co., St. Louis, Mo.) as the only nitrogen source and 0.5% YCB, was found to cause optimal elastase and collagenase production.

Culturing of *A. fumigatus* in Sabouraud-glucose medium used for comparison was performed as previously described.¹² Briefly, precultures of *A. fumigatus* were grown in 500 ml Erlenmeyer flasks, containing 150 ml Sabouraud's growth medium containing 2% glucose. The flasks were inoculated with 0.1 ml spore suspension containing 100 to 200 spores in 0.5% Tween 80. After 3 days of culturing in a New Brunswick incubator (New Brunswick Scientific Co., Inc., Edison, N.J.) at 37° C and 110 rpm, small mycelial spheres were harvested. Ten mycelial spheres from these precultures were inoculated into 2 L Erlenmeyer flasks containing 1 L Sabouraud-glucose medium and cultured as described above.

Collagen cultures

For protease production 1 L of 0.2 mol/L borate buffer containing collagen (0.2%) and YCB (0.5%) (modified from Kothary et al.⁶) in 2 L Erlenmeyer flasks were inoculated with 1.0 ml of mycelial particles from *A. fumigatus* precultures, which had been gently disrupted to obtain optimal distribution of germinating centers in the inoculated growth medium. The cultures were incubated at 37° C with shaking (150 rpm) for 5 days in a New Brunswick incubator. After 5 days of culturing no visible (insoluble) collagen was left, and culture filtrates were isolated.

Preparation of antigens

Preparation of Sabouraud-glucose-derived culture filtrates (L-SCF, E-SCF) and collagen-YCB-derived culture filtrates (E-CCF) used for comparative studies was performed as previously described for Sabouraud-glucose culture filtrates.¹⁴ Briefly, the mycelia were separated from the culture medium by Buchner filtration with double Whatman 541 filters (Whatman Ltd., Maidstone, England) followed by Seitz filtration with cellulose acetate filters (Sartorius AG, Goettingen, Germany) with successive pore sizes of 8.0 μ m, 3.0 μ m,

0.8 μm , and finally 0.45 μm . The Sabouraud-glucose culture filtrate was dialyzed for 18 hours against streaming tap water and concentrated to a final volume of 100 ml with an Amicon hollow fiber system (DC2) (Amicon B.V., Oosterhout, The Netherlands), with the use of H1P3-20 hollow fiber cartridges. The concentrate was lyophilized. The collagen-YCB culture filtrate was concentrated by ultrafiltration (YM10 filter, Amicon) and lyophilized, yielding the E-CCF. L-SCF was isolated after 40 days of cultivation, whereas E-SCF was isolated after 7 days of cultivation.

Chemical composition

Protein concentration was determined by the method of Lowry et al.¹⁶ with a bovine serum albumin (Sigma) standard. Glycan differentiation for characterization of carbohydrate structures on E-CCF proteins, bound to nitrocellulose was done with the use of a digoxigenin glycan differentiation kit (Boehringer Mannheim GmbH, Mannheim, Germany).

Sodium dodecylsulfate–polyacrylamide gel electrophoresis

Culture filtrates were separated on sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli.¹⁷ Protein samples were boiled in sample buffer (2% SDS, 5% 2-mercaptoethanol, and 10% glycerol in 25 mmol/L Tris-HCl; pH 6.8). E-CCF (0.72 mg) and E-SCF (1.25 mg) and L-SCF (1.25 mg) proteins were electrophoresed at room temperature (200 V) in a 1.5 mm, 12.5% polyacrylamide slab gel (Mini-Protean 11 Dual Slab Cell; Bio-Rad, Rockville Centre, N.Y.) with a reservoir buffer consisting of 25 mmol/L Tris-HCl (pH 8.3), 192 mmol/L glycine, and 0.1% SDS. Separated proteins were fixed with a 10% methanol–10% acetic acid solution and subsequently stained with Coomassie Brilliant Blue R250 (Merck, Darmstadt, Germany). Estimation of apparent molecular mass was based on commercially prepared standards (Pharmacia, Uppsala, Sweden). Proteins in duplicate, unfixed, unstained gels were used for Western blotting.

Immunochemical procedures

Double immunodiffusion according to the Ouchterlony method with the macroassay¹⁸ and IgG ELISA with a single-layer assay (immobilized antigen/human IgG (serum)/goat anti-human IgG-alkaline phosphatase) were performed as previously described.¹⁹ ELISA plates ("high binding"; Greiner Labortechnik, Alphen a/d Rijn, The Netherlands) were coated with antigens in 1 mg/ml NaHCO_3 , pH 9.6, and serum dilutions of 1:250 were used as previously described.¹⁸ Photometric analysis at 405 nm was recorded in duplicate, and the mean of two readings was calculated and plotted. Specificity of the assay is defined as the ratio of the photometric extinction found at 405 nm after correction for blank values, with pooled sera from patients

with ABPA or PA over the extinction found with pooled sera of healthy control subjects.

Western blot analysis was done according to standard procedures. Proteins separated by SDS-PAGE were transferred electrophoretically (Mini Transblot System, Bio-Rad) onto nitrocellulose paper (Bio-Rad) in cooled 25 mmol/L Tris buffer, pH 8.3, containing 192 mmol/L glycine, 0.05% SDS, and 10% methanol. Nitrocellulose sheets were incubated for 3 hours at room temperature with patient serum dilutions of 1:650 in Phadezym RAST buffer (Pharmacia). Between incubations, the blots were washed with three changes of washing buffer (for 5 minutes each, with shaking).²⁰ Goat anti-human IgG alkaline phosphatase conjugate (ICN ImmunoBiologicals, Zoetermeer, The Netherlands) was diluted 1000 times in Phadezym RAST buffer, and nitrocellulose sheets were incubated for 2 hours at room temperature with gentle shaking. Substrate (5-bromo-4-chloro-3-indolyl phosphate and nitro tetrazolium blue solution, available as one component from Kirkegaard & Perry Laboratories Inc., Gaithersburg, Md.) was added undiluted for a few minutes until color developed. The reaction was stopped by flooding the nitrocellulose sheets with distilled water.

Quantification of elastase activities

A colorimetric assay (modified from the method of Shotton²¹) with elastin–Congo red (Sigma) as the substrate was used to quantify elastase. Five hundred microliters of 0.2 mol/L sodium borate (pH 8.0), 500 μl of elastin–Congo red (20 mg/ml) in the same buffer, and 500 μl of test sample were mixed in a 3 ml centrifuge tube. After 45 minutes of 37° C, insoluble material in the assay mixture was removed by centrifugation at 0° C, and the extinction of released dye in the supernatant was measured spectrophotometrically at 495 nm. Standard curves were constructed by measuring the absorbance of dilutions of elastin–Congo red, which had been completely solubilized with porcine pancreatic elastase (Sigma). One unit of elastase activity is the amount of enzyme catalyzing the release of soluble dye equivalent to 1 mg of elastin–Congo red in 45 minutes at 37° C. For each test sample a control test sample was prepared by heating a test sample–borate mixture (without elastin–Congo red added) for 30 minutes at 60° C to inactivate enzyme activity. Before centrifugation at 0° C, the substrate was added to the inactivated enzyme mixture. The supernatant served as blank value for the test samples.

Quantification of collagenolytic activity: Orange collagen assay

Ten milligrams of orange collagen (kindly donated by Dr. Greetje H. Vos-Scheperkeuter) was suspended in 0.9 ml of 0.05 mol/L Tris buffer, pH 7.5, containing 5 mmol/L $\text{Ca}(\text{CH}_3\text{COO})_2$. Enzyme solution (0.02 ml) was added and incubated, while shaking at 37° C for 30 minutes. The reaction was stopped by addition of 0.1 ml

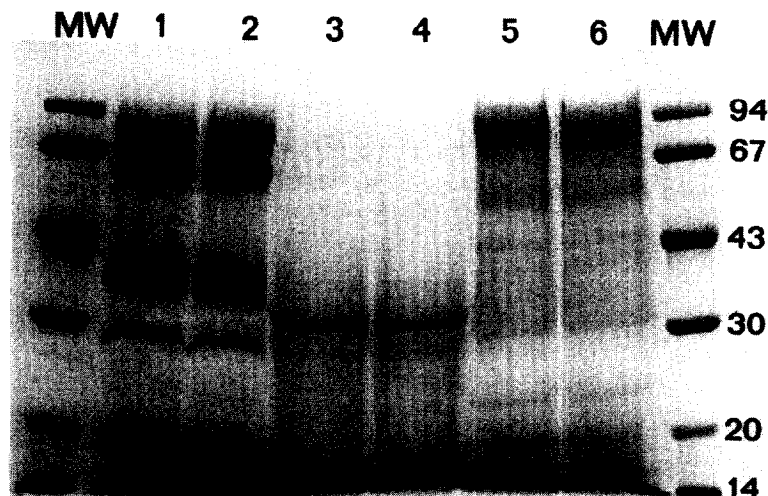


FIG. 1. SDS-PAGE of E-CCF (third and fourth lanes) compared with E-SCF (first and second lanes) and L-SCF (fifth and sixth lanes). MW, Molecular weight markers. Proteins were stained with CBB.

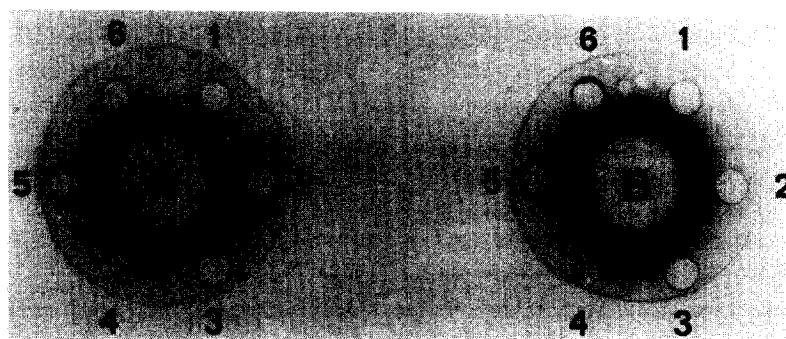


FIG. 2. Precipitates against different culture filtrates of *A. fumigatus* with double immunodiffusion with different patient pooled sera. **A**, Pooled sera of patients with ABPA. **B**, Pooled sera of patients with PA. 1,3, E-CCF; 2, L-SCF; 5,6, E-SCF; 4, not used.

of 28 mmol/L ethylenediaminetetraacetic acid (EDTA) solution. The mixture was then centrifuged for 10 minutes at 300 rpm, and the optical density of the supernatant was measured at 490 nm versus the blank. The blank was obtained by addition of enzyme solution to the mixture of substrate and EDTA and treating it in the same manner. One unit is defined as the amount of collagenolytic activity that hydrolyzes 1 nmol orange collagen per second.

In addition, enzyme activity for the synthetic substrate Forylacryloyl-Leu-Gly-Pro-Ala (Sigma) was performed according to the method of Van Wart et al.²²

Zymogram procedures were performed according to the method of Heussen and Dowdle²³ with a continuous 7.5% polyacrylamide gel with copolymerized 0.1% gelatin. Samples of 2.5 and 25 μ g in 10 μ l stacking gel buffer (50 mmol/L Tris-HCl, pH 6.8) were mixed with 10 μ l of sample buffer and electrophoresed at 4° C. Samples were mixed with sample buffer immediately before electrophoresis. Samples were not boiled.

Protease activity

Protease activity was assayed according to the method of Wolters et al.²⁴ by measuring the hydrolysis of casein (Difco). After incubation of a 10 to 40 μ l sample with 0.5 ml of 1% (wt/vol) casein in 100 mmol/L Tris-HCl, 5 mmol/L CaCl₂ (pH 7.4) during 30 minutes at 37° C, 0.7 ml of 13% (wt/vol) trichloroacetic acid was added, and samples were centrifuged at 1000 g for 10 minutes. Absorbance was read at 280 nm with casein as a standard. One unit of protease is the amount of enzyme that hydrolyzes 1 mg casein in 30 minutes.

Identification of elastase activity: Column chromatography

E-CCF antigens in 0.15 mol/L NH₄HCO₃ buffer (pH 8.1) (150 mg dry weight per 5 ml) were separated by means of Superdex 75 (Fast Flow) Hiload 26/60 (preparative grade) column chromatography (Pharmacia) with a fast-performance liquid chromatography system

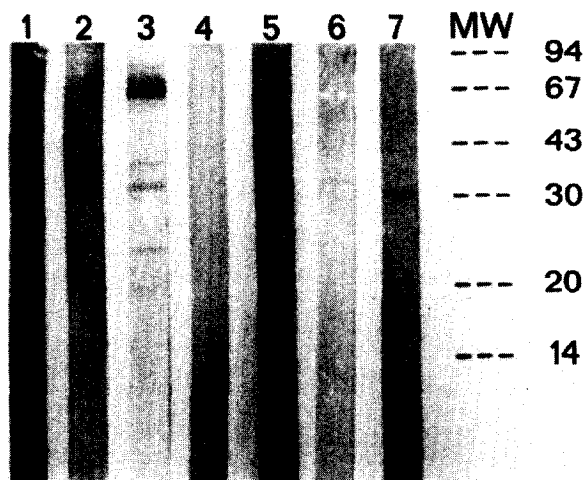


FIG. 3. Glycoprotein differentiation test on E-CCF. Lanes 2 (concanavalin A) and 3 (*Galanthus nivalis* agglutinin) show positive binding of 20, 25, 32, 35, and 67 kD proteins. Lanes 1 (wheat germ agglutinin), 4 (*Sambucus nigra* agglutinin), and 6 (*Maackia amurensis* agglutinin) are negative, whereas lane 5 (*Datura stramonium* agglutinin) and lane 7 (peanut agglutinin) bind the 32 kD protein.

(Pharmacia). Molecular weight markers (Pharmacia) were used in a separate run to calibrate the column. The proteins were eluted from the column at 2.5 ml/min, and fractions of 15 ml were collected. The fractions were tested for elastase activity.

E-CCF antigens (400 mg dry weight) were also applied to a cation-exchange S-Sepharose (Fast Flow) column (type XK, Pharmacia) in 3 ml of 50 mmol/L 2-morpholinoethanesulfonic acid-NaOH (Aldrich-Chemie, Steinheim, Germany), pH 5.5, with a fast-performance liquid chromatography system. Bound proteins were eluted from the column at 6.0 ml/min with 2-morpholinoethanesulfonic acid-NaOH/250 mmol/L NaCl buffer, pH 5.5. Bound and unbound fractions were tested for elastase activity. Fractions were concentrated (10 times) with Centricon-3 concentrators (Amicon). The concentrated samples were submitted to SDS-PAGE.

RESULTS

Culture of *A. fumigatus* on collagen-YCB medium

Using the inoculation procedure that is routinely used for culture in Sabouraud-glucose medium (i.e., 10 to 100 spores/flask), we found that spores of *A. fumigatus* from solid Sabouraud agar precultures were only incidentally able to grow on elastin-YCB. Only after inoculation of elastin-YCB media with high numbers of spores ($>10^8$), did some spores germinate and form mycelial spheres. This indicates that only small numbers of spores of this *A. fumigatus* strain were able to

produce the relevant enzyme(s) for the degradation of elastin. One mycelial sphere was used for further culture.

When media containing collagen instead of elastin were inoculated with the mycelia from the elastin cultures, rapid growth of the fungus and degradation of collagen were observed, suggesting that proteolytic enzymes, which were able to degrade both elastin and collagen, were produced by this strain. Optimal elastase production (0.080 U/mg protein) in the culture filtrate was found in cultures with 0.2% collagen supplemented with 0.5% YCB in 0.2 mol/L borate buffer. No elastase activity was found in parallel Sabouraud-glucose culture media (0.000 U/mg).

Protein profiles of E-CCF, L-SCF, and E-SCF were found to be different after separation on SDS-PAGE and CBB staining (Fig. 1). An intense protein band was found in E-CCF at 32 kD, which was not found in E-SCF or L-SCF. Fewer protein components are found in E-CCF than in E-SCF and L-SCF.

Double immunodiffusion patterns of the E-CCF, L-SCF, and E-SCF showed intense precipitations with the pooled sera of both patients with PA and those with ABPA (Fig. 2). Fewer precipitating components were found for ABPA pooled sera than PA pooled sera. With the pooled sera of patients with PA, precipitates of E-CCF versus E-SCF (wells 1 and 6) and E-CCF versus L-SCF (wells 1, 2, and 3) showed antigenic components with total identity, partial identity, and nonidentity, indicating the presence of antigens in E-CCF not shared with L-SCF or E-SCF.

Glycoprotein staining of the E-CCF with different lectins showed recognition of the 32 kD protein with both concanavalin A and *Galanthus nivalis* agglutinin (Fig. 3). Also, proteins found at 20, 25, 40, and 67 kD recognized both lectins, indicating the presence of mannose-containing carbohydrate structures or N-glycosidically linked "high mannose" or "hybrid-type carbohydrate chains. The 20, 25, 40, and 67 kD proteins stained only faintly with CBB (Fig. 1). These data suggest that these proteins are highly glycosylated.

After SDS-PAGE of E-CCF, L-SCF, and E-SCF, proteins were transferred to nitrocellulose sheets. IgG immunoblotting with pooled sera from patients with ABPA and patients with PA showed IgG binding to the 32 kD band found uniquely in E-CCF (Fig. 4). When pooled sera from healthy control subjects ($n = 45$) was used, binding to the 32 kD protein in E-CCF was very weak. Individual sera of the healthy control sub-



FIG. 4. Western blot analysis of *A. fumigatus* antigens. **A and B**, E-CCF (third and fourth lanes) compared with E-SCF (first and second lanes) and L-SCF (fifth and sixth lanes). *MW*, Molecular weight markers. **A**, IgG immunoblot with pooled sera of patients with ABPA. **B**, IgE immunoblot with pooled sera of patients with PA.

jects showed weak binding to the 32 kd protein in 12 of the 45 cases. The remaining 33 sera did not stain at all with the 32 kd protein (not shown). All individual sera from patients with PA ($n = 10$) and ABPA ($n = 10$) showed strong binding to the 32 kd protein (data not shown).

Other antigenic components found exclusively in E-CCF showed differential binding of IgG between patient groups. IgG binding was found with a 94 kd protein with pooled sera from patients with PA only (Fig. 4). Individual sera from patients with PA showed binding to the 94 kd protein in eight of 10 cases. Binding to this protein was found with individual ABPA sera in only two of 10 cases. A band at 67 kd was observed to bind IgG from pooled sera of patients with ABPA only. This band bound IgG in sera of individual patients with ABPA in three of 10 cases. Such binding was seen only with one PA serum sample.

Higher antibody levels were found in both ABPA and PA pooled sera by IgG ELISA with the routine E-SCF and L-SCF than with E-CCF. The binding specificity in the IgG ELISA was lowest for E-SCF and highest for E-CCF: the ratio for pooled ABPA sera over pooled healthy control sera was 1.4 for E-SCF, 3.9 for L-SCF, and 7.3 for E-CCF; and these ratios were 1.3, 3.9, 5.5, respectively, for pooled PA sera. IgG ELISA levels with individual sera from both patient groups (Fig. 5, *A* and *B*) generally showed a pattern similar to that of the pooled sera (i.e., lower levels and higher specificity in IgG ELISA when E-CCF is used compared with both L-SCF

and E-SCF). The exception was one individual with ABPA who showed a higher titer with the E-CCF (Fig. 5, *A*).

Elastase activity was high in E-CCF (0.14 U/mg protein) and was low or totally absent in L-SCF (0.007 U/mg protein) and E-SCF (0.0 U/mg protein). Collagenase activity to the specific bacterial collagenase substrate Furfurylacryloyl-Leu-Gly-Pro-Ala was absent from all culture filtrates tested, indicating nonidentity with bacterial collagenase activity. Collagenase activity to collagen (as quantified by the orange collagen assay) was relatively high for E-CCF (10.9 mU/mg protein) and low for E-SCF (0.008 mU/mg protein) and L-SCF (0.024 mU/mg protein). Remarkably, EDTA only partially inhibited enzyme activity in the orange collagen assay (approximately 40%), suggesting the presence of different types of enzymes, some of which are not inhibited by EDTA. Total protease activity (as quantified by casein digestion) was high for E-CCF (2.9 U/mg protein) and low for E-SCF (0.1 U/mg protein) and absent from L-SCF (0.0 U/mg protein).

The gelatin zymogram on E-CCF, E-SCF, and L-SCF, which was performed under nondenaturing conditions, showed a high molecular weight band (>200 kd) in E-CCF with high gelatin-degrading activity (data not shown). No gelatin-degrading activity was found on E-SCF and L-SCF.

Elastase activity was assayed in E-CCF protein components separated by fast-performance liquid chromatography gel filtration on Superdex 75 (FF) and cation-exchange on S-Sepharose (FF).

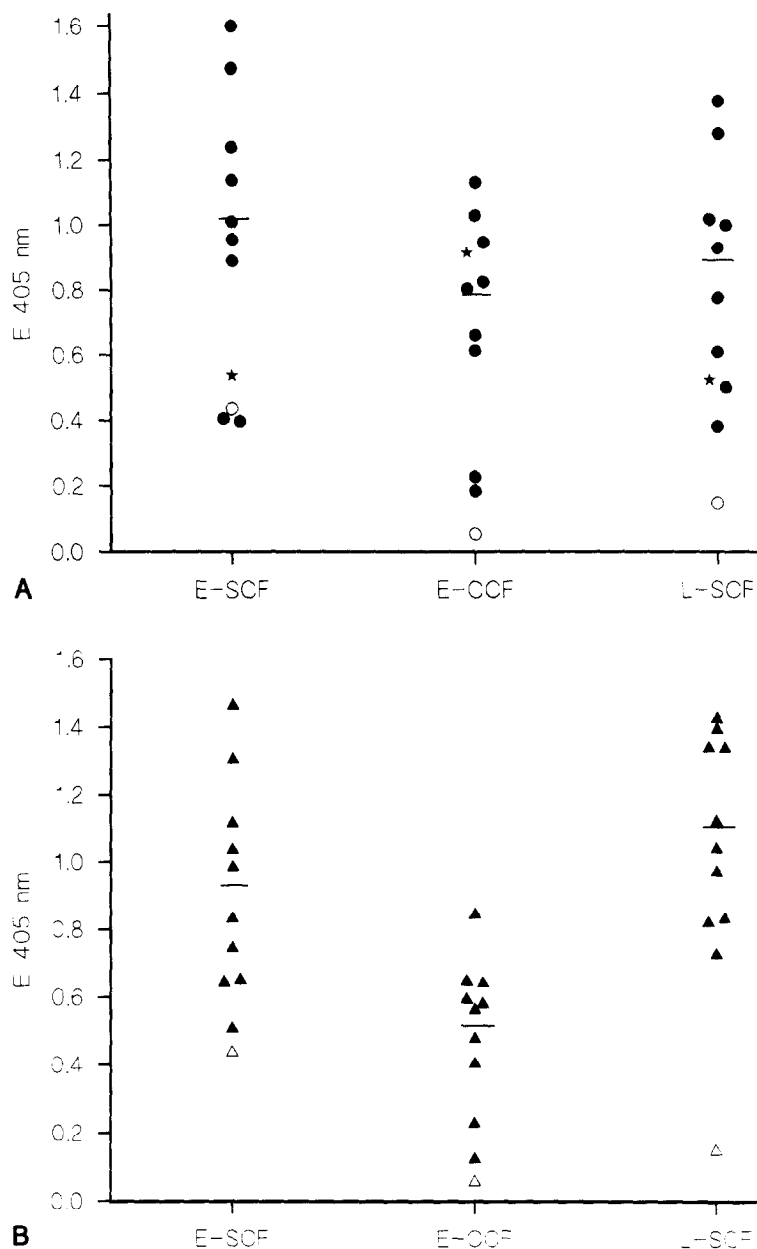


FIG. 5. A, IgG ELISA antibody levels against *A. fumigatus* culture filtrates with individual sera from patients with ABPA. The various symbols represent the extinctions found at 405 nm with the individual sera. Serum dilutions ($\times 250$) are used. *Star*, Patient showing high IgG binding to E-CCF compared with E-SCF and L-SCF (see text). *Open symbols*, pooled serum from healthy control subjects. **B,** IgG ELISA antibody levels against *A. fumigatus* culture filtrates with individual sera from patients with PA. The various symbols represent the extinctions found at 405 nm with the individual sera. Serum dilutions ($\times 250$) are used. *Open symbols*, Pooled serum from healthy control subjects.

The fractions eluted from the gel filtration column with an estimated molecular weight range of 25 to 40 kd (Fig. 6) correlated with elastase activity (1.12 U/mg protein) and yielded a band at 32 kd and minor bands at 25 and 10 kd on SDS-PAGE (data not shown). The bound fractions eluted from the S-Sepharose column with 250 mmol/L NaCl contained elastase activity (0.39

U/mg protein) and yielded a single 32 kd band on SDS-PAGE (Fig. 7). The unbound fraction contained elastase activity with lower specific activity (0.18 U/mg protein) and showed only a faint 32 kd band on SDS-PAGE. Thus elastase activity coisolated with a protein band at 32 kd, suggesting that the elastase activity may be associated with this 32 kd moiety.

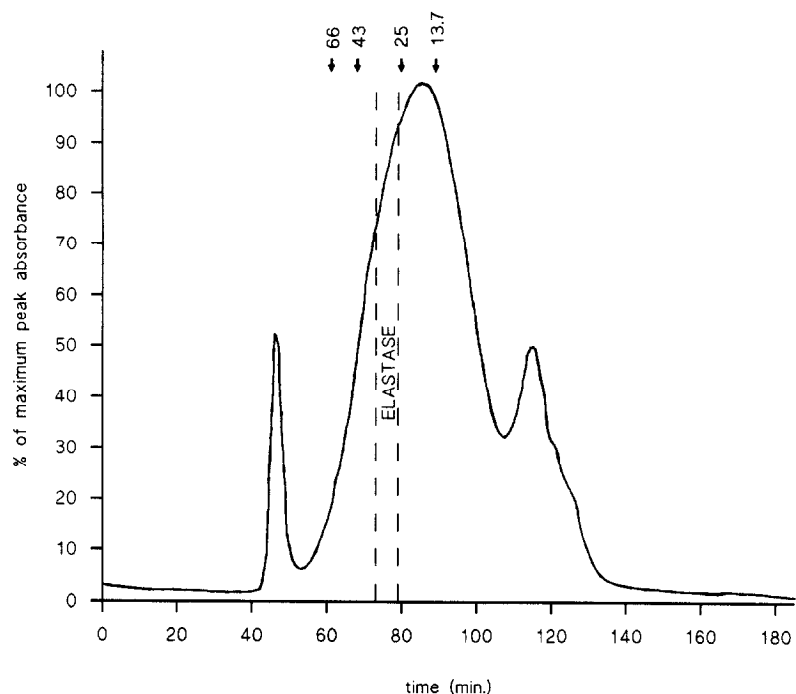


FIG. 6. Gel filtration chromatography of E-CCF on Superdex 75. One hundred fifty milligrams (dry weight) of lyophilized E-CCF was suspended in 5 ml of 0.15 mol/L ammonium bicarbonate buffer (pH 8.1) and filtered through a 0.2 μ m filter to remove insoluble materials. The cleared suspension was applied to an XK 26/60 column (Hiload, Pharmacia) containing 300 ml of the matrix, which had been previously equilibrated with the same buffer. Molecular weight markers are shown.

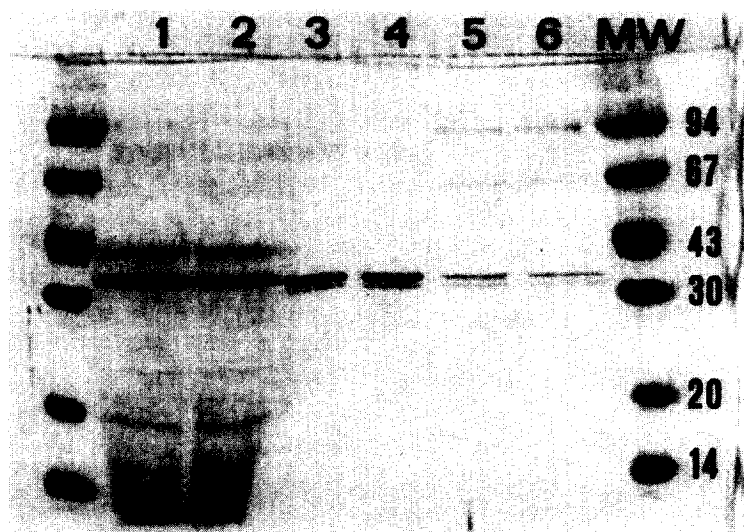


FIG. 7. SDS-PAGE of E-CCF (first and second lanes), E-CCF fractions after cation-exchange chromatography with high specific enzyme activity (bound fraction, third and fourth lanes) and low specific enzyme activity (unbound fraction, fifth and sixth lanes). MW, Molecular weight markers. Proteins were stained with CBB.

DISCUSSION

Fungi have been known to produce extracellular enzymes for some time.²⁵ Several extracellular proteolytic enzymes from *A. fumigatus* have been

described,^{26, 27} but interest in the possible role of these enzymes in the pathogenicity of aspergillosis has increased only in recent years. The importance of elastase to the pathogenicity of *A. fumi-*

gatus was demonstrated by Kothary et al.⁶ who showed that all mice exposed to elastase-producing strains of *A. fumigatus* died within 48 to 96 hours, whereas when exposed to nonelastase-producing strains, only 9 of 24 mice died. It has also been shown that clinical isolates of *Aspergillus* species, which cause an invasive aspergillosis, produce elastase activity during cultivation.⁷ However, the importance of elastase in invasive aspergillosis has also been questioned. In a study by Denning et al.²⁸ no overall loss of elastic tissue was found in blood vessel walls infiltrated by hyphae of *A. fumigatus*. The authors concluded that elastolysis of vessel walls, if significant, was just localized and/or transient. Antibodies against fungal proteases have never been demonstrated in patients with aspergillosis.

For the determination of antibodies against *A. fumigatus* in sera of patients with aspergillosis, antigens that are derived from cultures that have been grown on synthetic media are used. Generally these media do not contain matrix tissue proteins. Because microorganisms adapt themselves to culturing conditions, the media that are generally used will not promote the induction of tissue-degrading proteolytic enzymes such as elastase and collagenase. Therefore we studied the immunologic significance of a culture filtrate obtained on a collagen-containing medium with an *A. fumigatus* strain originally isolated from a patient with PA¹⁴ and compared this with routine culture filtrates of *A. fumigatus* cultured on standard Sabouraud medium.

It was found that only a limited number of spores produced by this strain were able to grow on media with either elastin or collagen as the sole protein source, whereas abundant growth was observed with much lower spore numbers under routine Sabouraud conditions. These findings are in agreement with the observation of Rhodes et al.⁷ that the percentage of clinical isolates of *A. fumigatus* producing elastase was similar to the limited numbers obtained from environmental isolates. Our results are thus probably due to adaptation of the *A. fumigatus* strain on elastin-YCB medium by selection of spores with a natural phenotype able to grow on collagen- or elastin-containing medium. When growth was obtained on the collagen or elastin media, the rate of growth was similar to that obtained on the Sabouraud media. High levels of elastase and collagenase were found in filtrates from collagen-

containing medium but not in filtrates from standard Sabouraud media.

The 32 kd glycoprotein in E-CCF coisolated with elastase activity, and this association was maintained with two different isolation methods. In the studies of Frosco et al.⁹ and Monod et al.³ a similar *A. fumigatus* exoprotease was generated when *A. fumigatus* was cultured on elastin-containing and soluble collagen-containing media, respectively. This elastase had an apparent molecular mass of 32 to 33 kd as determined by SDS-PAGE but showed a mass of 19.1 kd⁹ as determined by gel filtration. In our study a glycoprotein band at 32 kd was identified both by gel filtration chromatography and SDS-PAGE, which is probably related to the enzyme described by Frosco et al.⁹ and Monod et al.³ In the study of Monod et al.,³ the enzyme possessed both elastolytic and collagenolytic activity and was identified as a serine protease closely related to an alkaline protease derived from *A. oryzae*. In contrast to our findings, the elastase identified by Frosco et al.⁹ showed no carbohydrate binding. It is possible that the periodic acid-Schiff reagent assay used by Frosco et al. was not sensitive enough for the determination of smaller carbohydrate moieties bound to proteins.

The immunologic relevance of *A. fumigatus* elastase was demonstrated by IgG antibody binding to the 32 kd glycoprotein in the IgG immunoblot experiments, with sera of patients with ABPA and PA. This indicates that this glycoprotein is probably produced and excreted during fungal growth in human lung tissue.

IgG binding to other (glyco)proteins not present in E-SCF and L-SCF was also observed. Pooled sera from patients with PA showed IgG binding to a 94 kd protein found only in E-CCF, which stained only faintly in SDS-PAGE (Fig. 1), did not bind to lectins (Fig. 3), but was identified on SDS-PAGE after partial purification by cation-exchange chromatography (Fig. 7). Thus despite its presence in only small quantities in E-CCF, this protein has great immunologic relevance in PA, showing intense IgG binding with pooled sera (Fig. 4) and 80% of individual sera from patients with PA. In contrast, it is of only minor immunologic significance in ABPA, in which binding with pooled sera (Fig. 4) and 80% of individual sera was virtually undetectable.

Conversely, a 67 kd glycoprotein produced by *A. fumigatus* only when grown in collagen-con-

taining medium seems to have more immunologic relevance to patients with ABPA than to those with PA, as illustrated by the differences in binding with pooled sera (Fig. 4). The reason for the relative specificity of these immune responses in PA and ABPA is unknown. It may be hypothesized that specific pathogenetic conditions may determine whether certain fungal proteins are produced or become accessible to the host's immune system. Alternatively, host factors may determine the specificity of the immune response, and this response may in turn relate directly or indirectly to the pathogenesis of the fungal disease in question. Such variability of fungal strains or host immune responsiveness may also explain in part why the striking serologic differences found with pooled sera are not uniformly encountered in individuals.

In summary, higher levels of fungal protease production are found when collagen-containing medium is used than when standard culture medium is used. Filtrates from collagen-containing medium contain (glyco)proteins not seen in filtrates from standard medium, and at least some of these (the 32, 67, and 94 kd components) are immunologically relevant and show high levels of IgG binding with sera from patients with ABPA and PA as compared with sera from healthy control subjects. When pooled sera were used, major serologic differences were found between PA and ABPA in that IgG binding to a 94 kd protein was seen only with sera from patients with PA and binding to a 67 kd glycoprotein was seen only with sera from patients with ABPA. Patterns in which individual sera were used showed the same trend with greater variability. A 32 kd glycoprotein, which coisolated with elastase activity, bound IgG from pooled and individual sera of patients with ABPA and PA, suggesting that this component is a fungal elastase that is produced in vivo and results in the generation of an antibody response in these patients.

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Prevention of allergic sensitization to β -lactoglobulin with conjugates made of β -lactoglobulin coupled to isologous immunoglobulin G

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We have shown that, under certain conditions, a single dose of a conjugate of β -lactoglobulin linked to isologous IgG suppresses specific antibody responses (IgE, IgG, and IgA) in rats immunized with β -lactoglobulin. This suppression is allergen specific. It also appears to be effective when animals are first immunized and then tolerized. In addition, administration of the β -lactoglobulin-IgG tolerogen abolishes mast cell mediator release. This has been shown both in vivo and with passively sensitized mast cells in vitro. We propose that the construction of a tolerogen made of whole protein allergen linked to IgG could provide a novel specific therapy for allergic reactions. (J ALLERGY CLIN IMMUNOL 1994;93:778-86.)

Key words: Allergy prevention, β -lactoglobulin, isologous IgG, tolerogen

Several immunologic strategies have been used either to prevent or treat allergy: chemical modification of allergens,¹ covalent linkage of allergens to nonimmunogenic carrier molecules,^{2, 3} and

Abbreviations used

β LG: β -Lactoglobulin
DSS: Disuccinimidyl suberate
PHG: Phosphate-N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid-gelatin buffer

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more recently, administration of immune complexes^{4, 5} or peptides alone.^{6, 7} Antigen presentation appears to be the key to determining the immunologic outcome. It is known that when