Evidence for Possible Involvement of an Elastolytic Serine Protease in Aspergillosis

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A number of isolates of *Aspergillus fumigatus* obtained from the hospital environment produced extracellular elastolytic activity. This activity was found to be catalyzed by a single 33-kDa protein which was purified and characterized to be a serine protease. *A. fumigatus*, when grown on the insoluble structural material obtained from murine and bovine lung, produced the same extracellular 33-kDa elastolytic protease, indicating that this enzyme is likely to be produced when the organism infects the lung. Polymerase chain reaction with an oligonucleotide primer based on the N-terminal amino acid sequence of the elastolytic enzyme yielded a cDNA which was cloned and sequenced. The active serine motif showed more similarity to subtilisin than to mammalian elastase. The amino acid sequence showed 80% identity to the alkaline protease from *Aspergillus oryzae*. Screening of hospital isolates of *Aspergillus flavus* showed great variation in the production of elastolytic activity and a much lower level of activity than that produced by *A. fumigatus*. The elastolytic protease from *A. flavus* was shown to be a serine protease susceptible to modification and inactivation by active serine and histidine-directed reagents. This protease cross-reacted with the antibodies prepared against the elastolytic protease from *A. fumigatus*. Immunogold localization of the elastolytic enzyme showed that *A. fumigatus* germinating and penetrating into the lungs of neutropenic mice secreted the elastolytic protease. An elastase-deficient mutant generated from a highly virulent isolate of *A. fumigatus* caused drastically reduced mortality when nasally introduced into the lung of neutropenic mice. All of the evidence suggests that extracellular elastolytic protease is a significant virulence factor in invasive aspergillosis.

Immunocompromised patients such as bone marrow transplant recipients are highly susceptible to invasive aspergillosis, an infection by the filamentous fungus aspergillus (5). The high mortality caused by this fungal infection is a major threat to long-term survival of such patients (33). Currently, toxic antifungal agents such as amphotericin B are used to treat patients with aspergillosis. Even with the best antifungal agents, mortality is extremely high, reaching as high as 94% (3, 9). There is an obvious need for an effective means to protect against and treat invasive aspergillosis, and such approaches could be directed against the virulence factors involved in this infection. However, virulence factors involved in invasive aspergillosis are not understood.

Filamentous fungi invade their hosts by using extracellular enzymes to degrade the structural barriers in the host. For example, phytopathogenic fungi secrete cutinase that hydrolyzes the insoluble biopolyester, cutin, that is the outermost barrier of plants (18, 19). The invading fungus subsequently penetrates the carbohydrate barriers by using extracellular enzymes (8, 12). Since barriers of animal tissues are composed of proteins, fungi would probably need proteases to invade them. Fungal spores enter immunocompromised patients mainly by inhalation. Since elastin, which constitutes about 28% of lung tissue, is a major structural component in the lung (38), the germinating fungi might need elastase to invade the lung. Experimental evidence for the production of

elastolytic enzyme(s) by *Aspergillus fumigatus* and *Aspergillus flavus* has been presented, and such elastases have been proposed to play a role in pathogenesis (20, 29, 31, 32). In this paper, we show that *A. fumigatus* and *A. flavus*, two opportunistic pathogens that cause invasive aspergillosis in immunocompromised patients, secrete elastolytic protease when grown on elastin. Purification and properties of the elastolytic proteases from the two organisms suggest that they both are serine proteases that show immunological cross-reactivity. The sequence of the cDNA for the elastolytic enzyme(s) from *A. fumigatus* shows a high degree of homology to previously studied alkaline proteases. We demonstrate that, when *A. fumigatus* is grown on the insoluble structural matrix from the lung, the same elastolytic protease is produced. Using immunogold cytochemical localization, we demonstrate the production of this extracellular protease in the germinating fungal conidia and hyphae in the host lung. We also show that a mutant deficient in this protease generated from a virulent isolate of *A. fumigatus* is much less virulent than the wild type in causing mortality in a murine model.

MATERIALS AND METHODS

Fungal culture conditions. *A. fumigatus* and *A. flavus* isolates included both environmental samples and isolates from patients. Isolates were maintained on YG agar plates (0.5% yeast extract, 2% glucose, 2 ml of trace elements per liter, and 1.5% agar). The extracellular media from 13 isolates of *A. fumigatus* and 28 isolates of *A. flavus* were monitored for elastolytic enzyme production. Conidia (10^7) of each isolate were used to inoculate 25 ml of medium, and equal aliquots (140 μl) of extracellular medium from each

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culture were assayed for elastolytic activity with Congo red-elastin after 3, 4, 5, and 6 days of growth. For enzyme purification, conidia were harvested from YG plates in water and 1 ml of the conidium suspension (5×10^7 conidia) was used to inoculate 100 ml of elastin-containing liquid medium in Roux bottles. This medium consisted of 1.17% yeast carbon base (Difco)–0.37% calcium carbonate–0.2% insoluble elastin (Sigma) for *A. fumigatus*, and the same medium without calcium carbonate was used for *A. flavus*. Cultures were grown at 37°C under stationary conditions, usually for 5 days.

Enzyme assays. Assays for elastolytic activity were done with Congo red-elastin or the peptide succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide as the substrate. Orcein-elastin and succinyl-Ala-Ala-Ala-*p*-nitroanilide were also tested as substrates. The substrates were from Sigma. The reaction mixtures contained the enzyme and 1 mM peptide *p*-nitroanilide in 20 mM Tris HCl, pH 8.0, in 0.8 ml (4), and the reaction rate was monitored by measuring the change in A_{410}. Elastolytic activity with Congo red-elastin was determined in 1 ml of 20 mM sodium borate buffer, pH 8.8, containing 5 mg of Congo red-elastin–enzyme. The reaction mixture was incubated at room temperature, and periodic A_{495} measurements were done as described by Shotton (36). Usually, assay conditions used were linear so that the 30-min time point represented a reliable enzyme activity level.

Elastolytic activity was also measured by determining the amount of radioactivity released after incubation of the enzyme preparation at 37°C with 1 mg of [^{3}H]elastin (0.2 μCi/mg), prepared as described before (1, 41) and kindly provided by Mark Wewers of The Ohio State University, in a total volume of 400 μl of water, adjusted to pH 8.0 with sodium hydroxide. The reaction mixture was centrifuged after various periods of incubation, and aliquots of the supernatant were assayed for radioactivity by liquid scintillation spectrometry.

For all inhibitor studies, the enzyme was preincubated for 30 min with the inhibitor and all other assay components except the substrate. After addition of the substrate, the enzyme activity was monitored as described above. Proteolytic activity was also measured with casein as substrate (34); collagenase activity was measured as described previously (42).

Purification of elastolytic protease from *A. fumigatus*. *A. fumigatus* was grown in liquid medium in 20 to 24 Roux bottles. The extracellular fluid (approximately 2,000 ml) was separated from the fungal mat by filtration through Miracloth (Calbiochem) and concentrated fivefold with a Pellicon (Millipore) concentrator with a PTGC membrane cassette (nominal molecular size cutoff: 10 kDa). Further concentration of the protein was done by sequential ammonium sulfate precipitations at 0 to 25% and 25 to 80% saturation. For each step, powdered ammonium sulfate was slowly added, the solution was stirred for 1 h at 4°C, and the precipitated protein was collected by centrifugation. The same protocol was used for the *A. flavus* enzyme.

A. fumigatus elastase was further purified by phenyl Sepharose chromatography. The ammonium sulfate (25 to 80%)-precipitated proteins were dissolved in 10 ml of 50 mM potassium phosphate buffer, pH 6.5, and applied to a phenyl Sepharose column (2.6 by 8 cm) equilibrated with 10% ammonium sulfate in water. After several bed volumes of 10% ammonium sulfate were passed, proteins were eluted with a linear gradient of 10 to 0% ammonium sulfate in a total volume of 800 ml. Since ammonium sulfate severely inhibits

the elastolytic activity, each fraction was dialyzed against water prior to assay with Congo red-elastin as the substrate.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli (21), with a 12.5% resolving gel and a 4% stacking gel. For protein sequencing and Western blots (immunoblots), the electrophoretically separated proteins were transblotted onto an Immobilon P polyvinylidene difluoride membrane (Millipore) and a nitrocellulose membrane, respectively.

Treatment of proteins with [^{3}H]DipF. Aliquots of the extracellular medium from *A. fumigatus* (150 μg) or *A. flavus* (180 μg) were incubated with [^{3}H]diisopropylfluorophosphate ([^{3}H]DipF) (New England Nuclear; 4.4 Ci/mmol, 16 μM) in 150 μl of 100 mM sodium phosphate buffer, pH 8.0, for 45 min at 24°C. The proteins were precipitated with trichloroacetic acid and collected by centrifugation. The protein pellet was washed with 3% trichloroacetic acid–diethyl ether to remove radioactive materials not covalently attached to the proteins; the pellet was dissolved by boiling it in Laemmli loading dye (21) and electrophoresed. The gel was stained with Coomassie blue, destained, treated with Fluorohance (Research Products International Corp.), dried, and autoradiographed.

Antibody production and Western blots. The SDS-PAGE gel band corresponding to the elastolytic protease was cut out, mixed thoroughly with Freund's adjuvant (complete for first injection, incomplete for subsequent injections), and injected subcutaneously into rabbits; two additional booster injections were made at 2-week intervals, after which the blood was collected by heart puncture. The immunoglobulin G fraction was purified from the serum by using a Gammabind G column (Genex Corp.) following the manufacturer's protocol. Western blots were done by the standard protocol (7), with the electrophoretic conditions indicated above and ^{125}I-protein A (New England Nuclear; 7 to 10 μCi/μg) and 5% nonfat dry milk as the blocking agent.

Molecular mass determination. Native molecular weight determinations were done by fast-protein liquid chromatography (FPLC) (Pharmacia) with a Superose 12 column (1 by 30 cm) with 50 mM NaCl in water as the solvent. The column was calibrated with a mixture of thyroglobulin (669 kDa), alcohol dehydrogenase-horse (80 kDa), bovine serum albumin (BSA) (69 kDa), carbonic anhydrase (29.7 kDa), cytochrome *c* (12 kDa), and vitamin B_{12} (1 kDa). Aliquots of the ammonium sulfate-precipitated protein from *A. fumigatus* and *A. flavus* were chromatographed. Molecular weight was also determined by SDS-PAGE with molecular weight standards.

Measurement of protein and radioactivity. Protein was measured with the Bio-Rad protein assay kit, based on the dye-binding assay of Bradford (6). Aliquots of solutions containing labeled materials were mixed with Scintiverse II BD (Fisher) and assayed for radioactivity in a Beckman LS3801 liquid scintillation counter.

Growth of *A. fumigatus* on insoluble polymeric material from lung. Bovine lung excised from a freshly killed animal was washed thoroughly with 100 mM NaCl, cut into small pieces with a razor blade, and blended in a Robot Coupe in 100 mM NaCl; the mixture was then homogenized with a Brinkman Polytron homogenizer. Freshly excised murine lungs were washed with 100 mM NaCl and homogenized directly in the Brinkman homogenizer. The insoluble material collected by centrifugation at $25,000 \times g$ for 10 min was rehomogenized and recentrifuged; the process was repeated twice. The pellet was homogenized in distilled water, the

insoluble material recovered by centrifugation was mixed thoroughly with a vortex mixer with an excess of a 1:1 mixture of chloroform and methanol, and after 1 h the insoluble material was recovered by centrifugation; the extraction was repeated twice. The final pellet was dried, and the resulting powder was used as the N source to replace elastin at a level of 0.2% in the medium containing yeast carbon source and calcium carbonate. The procedures used for inoculation, growth, and enzyme assays were the same as those used for elastin-grown cultures.

Generation of an elastolytic protease clone by PCR. A 20-mer oligonucleotide based on the N-terminal sequence of the protease was synthesized by using an Applied Biosystems 381A DNA synthesizer. Poly(A)$^+$ mRNA from *A. fumigatus* was isolated by guanidium thiocyanate-sarcosyl extraction followed by sedimentation through a CsCl solution (24) and used as a template for a polymerase chain reaction (PCR) technique which requires only a single gene-specific primer (10); the published protocol for 3' end amplification of cDNA was followed by using 250 pmol of 20-mer oligonucleotide as primer for the cDNA synthesis and altered amplification times: the 40-min annealing period at 72°C was decreased to 5 min, and 30 cycles of amplification were done at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The resulting PCR product was purified by electrophoresis, subcloned into the HindIII-EcoRI site of pUC19, and sequenced by the dideoxy chain termination method (35).

Preparation of elastase-deficient mutants of *A. fumigatus* by chemical mutagenesis. Spores of *A. fumigatus* were incubated with 100 μg of N-methyl-N'-nitro-N-nitrosoguanidine in 50 mM Tris-HCl, pH 7.5, with 0.1% Tween 80 for 1, 5, 10, 30, and 60 min. The conidia were collected by centrifugation, washed five times by resuspension and recentrifugation in water, and used to inoculate YG agar plates. Colonies were individually picked and grown in 1 ml of YG medium at 37°C. Within 48 h, a mat became visible and was then transferred to 2 ml of elastin medium and grown at 37°C for 24 h. Aliquots (20 μl) of the broth from these cultures were placed in wells in elastin plates (elastin medium, prepared with soluble, rather than insoluble, elastin, plus 1.5% agarose), and after 24 to 72 h, clearing zones were visible around wells of the elastase-producing colonies. The elastin media from colonies that did not produce a clearing zone were assayed enzymatically to quantitate the levels of elastase production in these mutants.

Measurement of virulence of *A. fumigatus* in a murine model. Six- to eight-week-old female BALB/c mice were housed in a Bioclean laminar flow hood and provided with water containing oxytetracycline (100 mg/liter)–neomycin sulfate (10 mg/liter), pH 2.5, from 7 days prior to irradiation to the end of the experiment. They were irradiated with 400 rads from a Gamma Cell 40 irradiator. Three days after irradiation, the mice became neutropenic. The degree of neutropenia was measured by using a hemocytometer to count the number of leukocytes and polymorphonuclear cells per milliliter of blood. For each time point, blood was drawn from the tail vein of 2 to 3 mice from each group and stained with Giemsa-Wright stain and cells were counted in triplicate samples. At 3.5 days, mice were inoculated intranasally with 10^8 *A. fumigatus* conidia suspended in 20 μl of 0.1% Tween 80 and then 10 μl of 0.1% Tween 80. Serial dilution plating on YG agar of homogenate of lung taken from animals sacrificed 30 min after the inoculation procedure showed that this method introduced 10^7 conidia into the mouse lung. The irradiated mice were injected subcutane-

ously every other day with 2 mg of cortisone acetate in 0.1% Tween 80, starting 8 to 12 h after inoculation with fungal conidia; nonirradiated mice were not injected.

Immunogold localization of elastolytic protease produced by *A. fumigatus* in the lungs of neutropenic mice. Lung tissue was sampled 0, 0.5, 1, 2, 3, 4, 6, 8, 12, 16, 20, and 24 h after inoculation of neutropenic mice with *A. fumigatus* conidia as described above. Lung tissue was fixed with 4% paraformaldehyde–0.5 glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (13). Samples were degassed under vacuum for 15 min and then placed at 4°C. After 4 h, the samples were washed four times for 15 min each with 0.1 M sodium phosphate buffer, pH 7.4, brought to 70% ethanol by a graded series, infiltrated with L. R. White embedding medium (28) diluted at 2:1, 1:1, and 1:2 with 70% ethanol for 1 h, 2 h, and overnight, respectively, and then changed three times in 100% L. R. White for at least 4 h each. Samples were placed in 00-size gelatin capsules with fresh embedding medium and polymerized at 60°C for 24 h (28). Blocks were cut with glass knives on a Reichert Ultracut Ultramicrotome. Silver-gold sections were placed on nickel 300-mesh grids (28) coated with Formvar (14). Immunolabeling was done according to Herman and Shannon (15). Coated grids with sections were immersed for 10 min in 1% BSA in 0.05 M Tris-buffered saline, pH 7.5, with 1% Tween 20. Grids were placed in diluted elastase antibody solution for 1 h, rinsed in Tris-buffered saline with Tween 20, and placed in 1% BSA for 5 min and then in goat anti-rabbit antiserum conjugated with 20-nm colloidal gold for 30 min before being washed in distilled water. Sections were stained with 8% aqueous uranyl acetate before being viewed with a Zeiss 10 electron microscope.

Nucleotide sequence accession number. The GenBank accession number for the primary nucleotide sequence for this enzyme is M99420.

RESULTS

Production of elastolytic protease activity by *A. fumigatus* isolates. To test whether *A. fumigatus* isolates found in the hospital environments to which immunocompromised patients are exposed produce elastolytic enzymes, a number of such isolates were grown on elastin-containing medium and the extracellular fluid was assayed for elastin-hydrolyzing activity. All of the isolates produced extracellular elastolytic activity, although there was much variability in the amount of activity produced (Fig. 1). The time course of production indicated that maximal levels of elastolytic activity were found in the medium after 5 days of growth and that subsequently the enzyme level began to decrease (data not shown). One isolate (no. 13) that produced the highest level of elastase was chosen for further studies.

Purification of elastolytic protease from *A. fumigatus*. The bulk of the elastolytic activity was recovered in the protein precipitated from the extracellular fluid between 25 and 80% saturation with ammonium sulfate. Upon administration of this enzyme preparation to a phenyl Sepharose column in 10% ammonium sulfate, all of the elastolytic activity was retained by the column. Elution of the protein by application of 10 to 0% ammonium sulfate yielded an elastolytic protease that emerged at a 3.5% ammonium sulfate concentration (Fig. 2A). Since ammonium sulfate severely inhibits this fungal elastolytic protease, each fraction had to be dialyzed before enzyme assays. Fractions containing the highest amounts of elastolytic activity were pooled, and the protease was recovered by 80% saturation with ammonium sulfate.
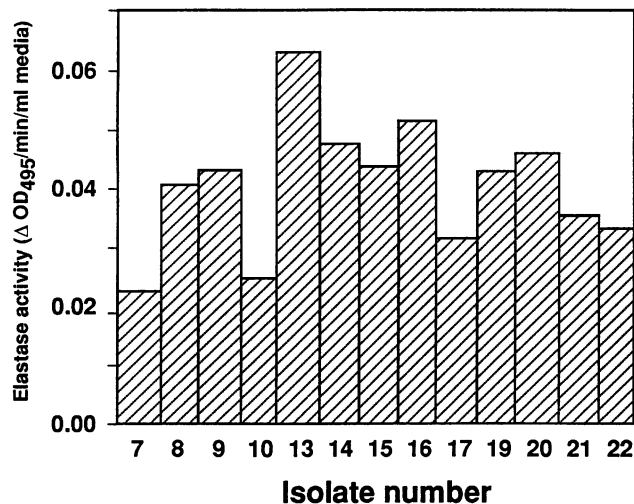
FIG. 1. Elastolytic protease production by hospital isolates of *A. fumigatus*, grown on an elastin-containing liquid medium. Activity levels were measured after 5 days of growth with Congo red-elastin as the substrate.

Because of self-digestion in solution, this protease was always stored as ammonium sulfate precipitate. SDS-PAGE of the enzyme preparation showed a single Coomassie blue-staining band corresponding to a molecular mass of 33 kDa (Fig. 2B), showing that this elastolytic protease was purified to near homogeneity. SDS-PAGE of the total extracellular fluid of elastin-grown *A. fumigatus* showed that the 33-kDa protein was one of the most abundant proteins in the culture filtrate (Fig. 2B). Therefore, it is not surprising that about 10-fold purification yielded a highly purified enzyme. FPLC gel filtration on Superose indicated that the protein had a smaller molecular mass of 22 kDa. Even though the properties of the protein that lead to the differences in apparent molecular weight are not understood, it appears clear that this protease is a single polypeptide. In support of this conclusion, N-terminal sequencing of the protein released a single amino acid in each cycle with >90% yield of the amino acid. This amino acid sequence is indicated later in the open reading frame revealed by the cDNA sequence.

Catalytic properties of the elastolytic protease from *A. fumigatus*. To characterize the catalytic properties of the enzyme, we used model substrate peptides, Congo red-elastin, and [³H]elastin as substrates. The enzyme showed maximal elastolytic activity between pH 7.0 and 8.0. Enzyme activity was sharply lower below pH 7.0 and above pH 8.0. Thiol-directed reagents such as *p*-hydroxymercuribenzoate and iodoacetamide had little effect on the enzyme activity (Table 1). Metal ion chelator *o*-phenanthroline even at 1 mM did not significantly inhibit this elastase, although EDTA showed some inhibition. However, the level of inhibition observed suggests that this fungal elastase is not a metalloprotease. Potato chymotrypsin inhibitor was an effective inhibitor of this fungal elastolytic protease. The enzyme was strongly inhibited by phenylmethanesulfonyl fluoride and DipF, suggesting that this elastolytic enzyme is a serine protease. In further support of this conclusion, the elastolytic activity could be titrated down to about 10% of the original activity by the addition of increasing concentrations of DipF (Fig. 3). The sensitivity to DipF was identical for hydrolysis of *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide or [³H]elastin, indicating that the same active site is involved
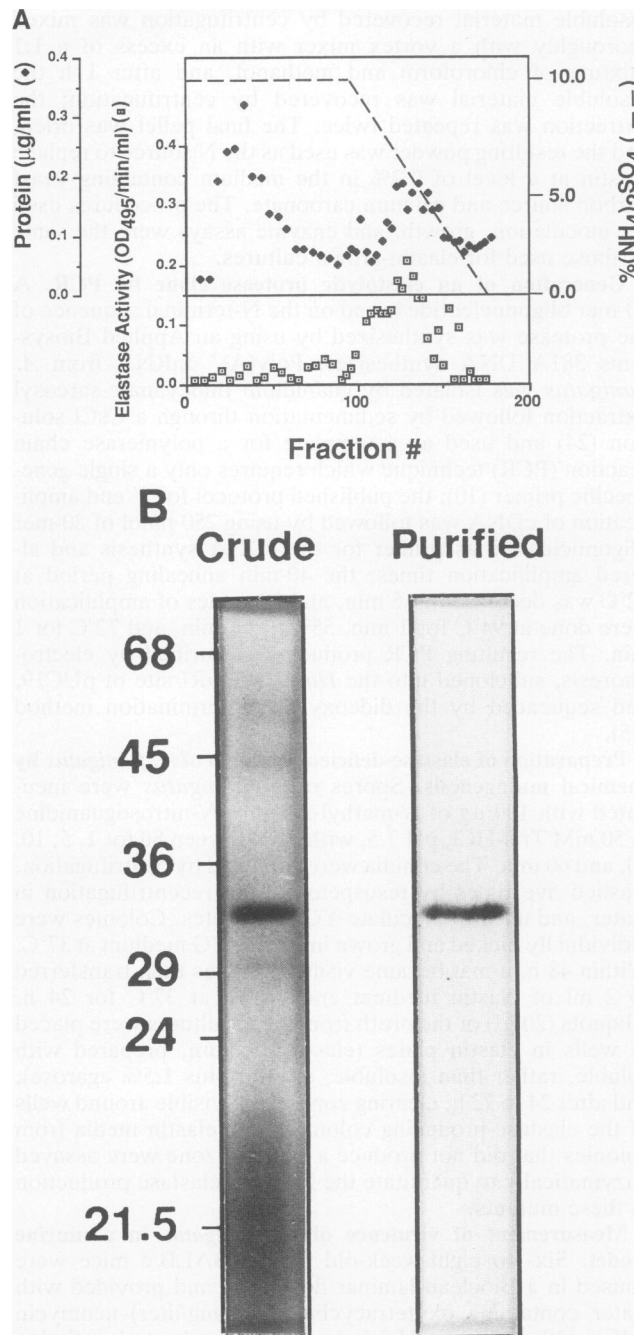

FIG. 2. (A) Purification of the elastolytic protease by chromatography on a phenyl Sepharose column. An enzyme preparation obtained by ammonium sulfate precipitation was administered to the column, and the enzyme was eluted by 10 to 0% ammonium sulfate gradient as described in Materials and Methods. Enzyme activity was measured with Congo red-elastin as substrate. (B) SDS-PAGE of the total protein from the extracellular fluid of *A. fumigatus* cultures grown on elastin medium (crude) and the purified elastolytic protease. Experimental details are in the text.

in the hydrolysis of both substrates. Serine proteases catalyze peptide hydrolysis with the catalytic triad involving serine, histidine, and a carboxyl group. To further test for the involvement of such a catalytic triad, the effect of a

TABLE 1. Effect of inhibitors on hydrolysis of Congo red-elastin by *A. fumigatus* elastase

Inhibitor	Concn	% Activity
No inhibitor		100
p-Hydroxymercuribenzoate	10 μM	93
	50 μM	94
Iodoacetamide	0.5 mM	100
	1 mM	86
EDTA	0.5 mM	80
	1 mM	61
DipF	0.05 mM	96
	0.1 mM	87
	0.5 mM	57
PMSF[a]	0.5 mM	58
	1 mM	46
o-Phenanthroline	0.5 mM	100
	1 mM	89
Potato chymotrypsin inhibitor	4 μM	78
	8 μM	57
	16 μM	39
	24 μM	28

[a] PMSF, phenylmethanesulfonyl fluoride.

histidine-modifying reagent, diethylpyrocarbonate (DEPC), on the elastolytic activity was tested. The fungal enzyme activity was increasingly inactivated with increasing concentrations of DEPC (Fig. 3). To further test for the presence of active serine in this enzyme, the protein preparation was treated with [³H]DipF and treatment was followed by SDS-PAGE and autoradiography. All of the ³H incorporated into the protein was found in the 33-kDa protein (Fig. 4). This result clearly shows that the 33-kDa protein is a serine protease.

A single elastolytic enzyme produced by A. fumigatus. To test for the possibility that the extracellular fluid contains more than one type of elastolytic enzyme, the total extracellular fluid from elastin-grown *A. fumigatus* was subjected to FPLC on Superose and all fractions were assayed for elastolytic activity. The only elastolytic activity was eluted as one peak corresponding to a molecular mass of 22 kDa (Fig. 5). When the total extracellular fluid was treated with [³H]DipF and subjected to SDS-PAGE and autoradiography, only one labeled band was observed, and this band corresponded to a 33-kDa protein as seen with purified elastase (Fig. 4). Western blot analysis with rabbit antiserum pre-

FIG. 4. Autoradiograms of SDS-PAGE of [³H]DipF-treated elastolytic protease from elastin-grown cultures of *A. fumigatus* and of the Western blot of the total extracellular proteins from the *A. fumigatus* culture fluid. Identical autoradiograms were obtained when total extracellular proteins or purified elastolytic protease was used for DipF treatment. Rabbit antibodies prepared against purified elastolytic protease from *A. fumigatus* and ¹²⁵I-protein A were used for the Western blot.

pared against the purified elastolytic protease from the culture fluid showed a single band corresponding exactly with the DipF-labeled (Fig. 4) and Coomassie blue-stained (Fig. 2B) band at 33 kDa. Thus, the extracellular fluid most probably contains only one elastolytic enzyme, and this enzyme is the only active serine-containing enzyme in the elastin-grown culture fluid.

Elastolytic enzyme production by A. fumigatus on the structural proteins from lung. It is postulated that when the fungus encounters the lung structural material during its infection of the animal, it would probably secrete elastolytic enzymes to penetrate such barriers to establish infection. To test whether the elastolytic protease produced when *A. fumigatus* is grown on elastin-containing medium is relevant to what happens when the organism contacts the lung structural material, *A. fumigatus* was presented with a crude preparation of the insoluble structural material obtained from mice lungs and bovine lungs. This material was prepared by thoroughly extracting the insoluble material recovered from lung homogenate with aqueous and organic solvents. The extracellular fluid showed elastolytic activity.
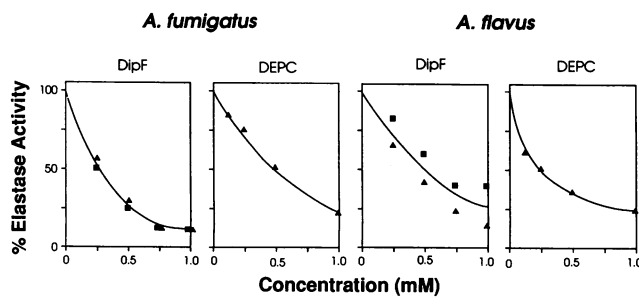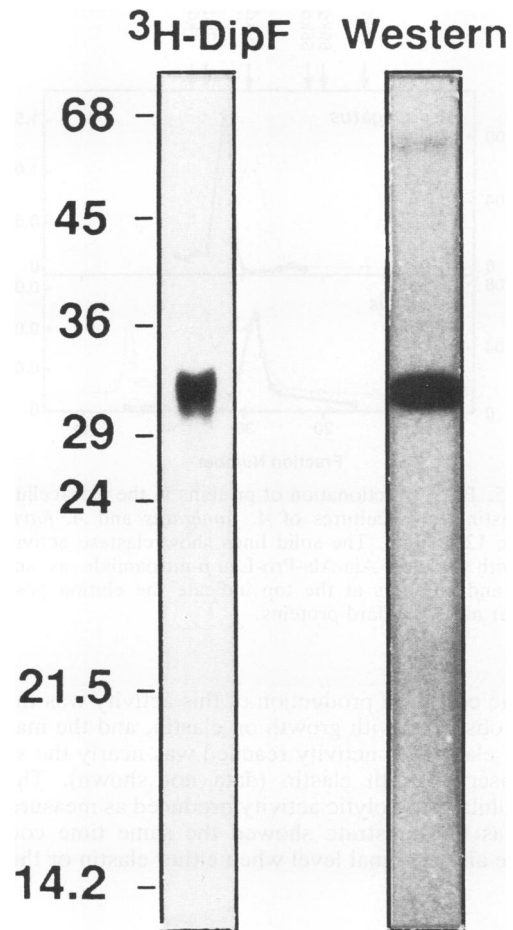

FIG. 3. Inhibition of the extracellular elastase activity from *A. fumigatus* and *A. flavus* by DipF or DEPC. Assays were done spectrophotometrically with N-succinyl-Ala-Ala-Pro-Leu-p-nitroanilide as the substrate (▲) with 40 (*A. fumigatus*) or 80 (*A. flavus*) μg of protein or with [³H]elastin as the substrate (■) with 12 (*A. fumigatus*) or 25 (*A. flavus*) μg of protein, as described in Materials and Methods.
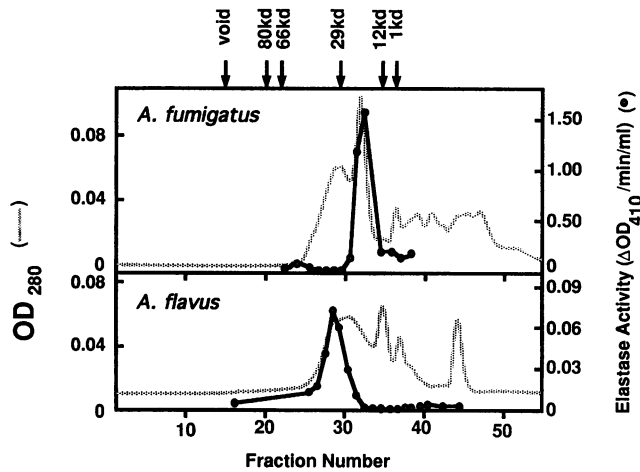
FIG. 5. FPLC fractionation of proteins in the extracellular fluid from elastin-grown cultures of *A. fumigatus* and *A. flavus* on a Superose 12 column. The solid lines show elastase activity measured with succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide as substrate. Arrows and numbers at the top indicate the elution position of molecular mass standard proteins.

The time course of production of this activity was the same as that observed with growth on elastin, and the maximum level of elastolytic activity reached was nearly the same as that observed with elastin (data not shown). The total extracellular proteolytic activity produced as measured with casein as the substrate showed the same time course of increase and maximal level when either elastin or the insol-

uble material from the lung was used as the nitrogen source. FPLC analysis showed that all of the casein-hydrolyzing activity was contained in the same fractions as those containing the elastolytic activity (Fig. 6A). No collagenase activity was detected in the extracellular fluid. SDS-PAGE of the extracellular fluid showed a significant Coomassie blue-staining band at 33 kDa exactly matching the elastolytic protease band generated by growth in elastin-containing medium. Western blot of the extracellular proteins from the culture grown on the lung material with the antiserum prepared against the protease obtained from the elastin-grown medium showed a strong band at 33 kDa (Fig. 6B) (Coomassie blue staining is not shown). Treatment of the extracellular fluid with [³H]DipF followed by SDS-PAGE and autoradiography revealed one band at 33 kDa. These results show that the elastolytic protease is one of the major proteins produced when *A. fumigatus* encounters the structural materials of lung tissue. The elastolytic enzyme produced under such conditions appears to be the same as that produced when the organism is grown with elastin. This enzyme appears to be the only serine protease produced by *A. fumigatus* when it encounters lung structural materials.

Structure of the elastolytic protease from *A. fumigatus*. To clone the *A. fumigatus* cDNA for the elastolytic protease, poly(A)⁺ mRNA from elastin-induced cultures was used to synthesize the first cDNA strand. With this strand as the template, PCR synthesis was done by using an oligo(dT) and a 20-mer oligonucleotide primer synthesized on the basis of the N-terminal amino acid sequence of the elastolytic enzyme as the primers (Fig. 7). Upon isolation, subcloning, and sequencing of the resulting 0.6-kb cDNA, an open reading frame with the sequence shown in Fig. 7 was found.
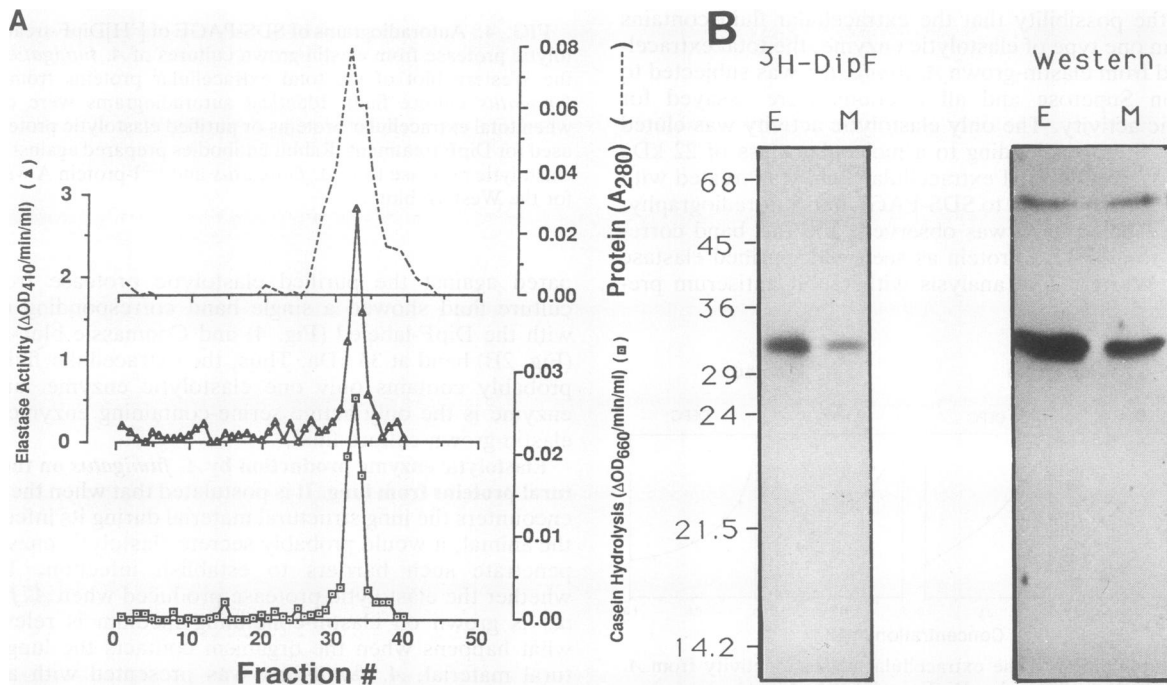

FIG. 6. (A) Elastolytic and casein-hydrolytic activities of the FPLC fractions of the proteins from the extracellular fluid of cultures of *A. fumigatus* grown on the insoluble structure matrix of murine lung. Experimental details are in the text. (B) Autoradiograms of SDS-PAGE of [³H]DipF-treated proteins (left) and of the Western blots of the total extracellular fluid of *A. fumigatus* cultures grown on elastin (E) and insoluble material from the murine lung (M). Rabbit antibodies prepared against purified elastolytic protease from *A. fumigatus* and ¹²⁵I-labeled protein A were used for the Western blot.

N-terminal sequenced residues (single underline):
his gln lys gly ala pro trp gly leu gly ser ile ser his lys gly gln ala ser

Pos																				
1	ACC	GAC	TAC	ATC	TAC	GAC	ACC	AGC	GCT	GGC	GCA	GGC	ACC	TAT	GCC	TAC	GTT	GTC	GAC	AGT
25	thr	asp	tyr	ile	tyr	asp	thr	ser	ala	gly	X	X	thr	X	ala	tyr	val	val	asp	ser
61	GGC	ATC	AAT	GTC	AAC	CAC	GTC	GAG	TTC	GAG	AGC	CGC	GCA	TCG	CTG	GCA	TAC	AAC	GCC	GCT
45	gly	ile	asn	val	asn	his	val	glu	phe	glu	ser	arg	ala	ser	leu	ala	tyr	asn	ala	ala
121	GGT	GGC	AGC	CAT	GTT	GAC	AGC	ATC	GGC	CAC	GGA	ACG	CAC	GTT	GCT	GGT	ACC	ATT	GGC	GGC
65	gly	gly	ser	his	val	asp	ser	ile	gly	his	gly	thr	his	val	ala	gly	thr	ile	gly	gly
181	AAG	ACC	TAC	GGA	GTG	GCC	AAG	AAG	ACC	AAC	CTT	CTG	TCC	GTC	AAG	GTC	TTC	CAG	GGC	GAG
85	lys	thr	tyr	gly	val	ala	lys	lys	thr	asn	leu	leu	ser	val	lys	val	phe	gln	gly	glu
241	TCC	CCT	AGC	ACC	TCC	ATC	ATC	CTT	GAC	GGC	TTC	AAC	TGG	GCT	AAT	GTC	GAC	ATT	GTG	AGC
105	ser	pro	ser	thr	ser	ile	ile	leu	asp	gly	phe	asn	trp	ala	asn	val	asp	ile	val	ser
301	AAG	GGT	CGT	ACT	AAG	AAG	GCT	GCG	ATC	AAC	ATG	AGC	CTT	GGT	GGT	GGT	TAC	TCT	TAT	GCC
125	lys	gly	arg	thr	lys	lys	ala	ala	ile	asn	met	ser	leu	gly	gly	gly	tyr	ser	tyr	ala
361	TTC	AAC	AAC	GCT	GTT	GAG	AAC	GCT	TTC	GAT	GAA	GGT	GTC	CTT	TCT	GTC	GTC	GCT	GCT	GGA
145	phe	asn	asn	ala	val	glu	asn	ala	phe	asp	glu	gly	val	leu	ser	val	val	ala	ala	gly
421	AAC	GAG	AAC	AGT	GAT	GCC	TCA	AAT	ACC	AGC	CTC	TTC	CGC	GTC	CTA	ACT	TTG	ACG	GTT	GCT
165	asn	glu	asn	ser	asp	ala	ser	asn	thr	ser	leu	phe	arg	val	leu	thr	leu	thr	val	ala
481	GCG	ATC	AAC	AAG	AGC	AAC	GCC	CGC	GCC	TCC	TTC	TCC	AAC	TAT	GGT	TCC	GTT	GTC	GAC	ATC
185	ala	ile	asn	lys	ser	asn	ala	arg	ala	ser	phe	ser	asn	tyr	gly	ser	val	val	asp	ile
541	TTC	GCT	CCC	GGT	CAG	ATA	ATC	CTT	TCG	GCC	TGG	ATT	GGC	TCC	ACC	ACT	GCC	ACC	AAC	ACC
205	phe	ala	pro	gly	gln	ile	ile	leu	ser	ala	trp	ile	gly	ser	thr	thr	ala	thr	asn	thr
601	ATC	TCC	**GGT**	**ACT**	**TCC**	ATG	GCC	ACC	CCT	CAC	ATT	GTT	GGC	CTA	TCC	GTC	TAC	TTG	ATG	GGT
225	ile	ser	**gly**	**thr**	**ser**	met	ala	thr	pro	his	ile	val	gly	leu	ser	val	tyr	leu	met	gly
661	CTT	GAG	AAC	CTC	TCT	GGC	CCT	GCT	GCA	GTG	ACC	GCT	CGC	ATC	AAG	GAG	TGG	GCC	ACC	AAT
245	leu	glu	asn	leu	ser	gly	pro	ala	ala	val	thr	ala	arg	ile	lys	glu	trp	ala	thr	asn
721	GGT	GTT	GTT	ACC	AAC	GTT	AAG	GCA	GCC	CCA	ACA	AGC	TTG	GCC	TAC	AAT	GGC	AAT	GCT	taa
265	gly	val	val	thr	asn	val	lys	ala	ala	pro	thr	ser	leu	ala	tyr	asn	gly	asn	ala	OCH

(The active serine motif — boxed in the figure — spans: gly thr ser met ala thr pro his ile val.)

```
                          C  C   A C C
        Primer : ACNGATTATATCTATGATAC
                                      T
```

FIG. 7. Partial nucleotide sequence and deduced amino acid sequence for the elastolytic protease from A. fumigatus. The N-terminal region of the mature protein was sequenced (single underline), and an oligonucleotide (double underline) with a redundancy of 192 was synthesized (shown at the bottom). This oligonucleotide was used as the primer in a PCR synthesis using A. fumigatus mRNA as the template, as described in Materials and Methods; N indicates that all four bases were used at that position. Unidentified residues are indicated by X. The active serine motif is indicated by the box.

The 5' sequence of the cloned cDNA matched the amino acid sequence of the protein as expected. Furthermore, additional amino acids identified towards the carboxyl side of the segment used for the oligonucleotide synthesis also matched the amino acid sequence deduced from the nucleotide sequence. This sequence differed by only a few nucleotides when compared with the sequences of protease genes from other isolates of A. fumigatus reported (16, 39) after the present sequence was obtained. Therefore, the PCR product probably represents the elastolytic protease of A. fumigatus. The amino acid sequence showed 80% identity to the alkaline protease previously obtained from Aspergillus oryzae (27).

Elastolytic protease from A. flavus. A. flavus is another species of Aspergillus that causes invasive aspergillosis (5). To test whether A. flavus produces an elastase as noted above for A. fumigatus, A. flavus species isolated from a hospital environment were tested for their ability to produce extracellular elastase. All the species tested produced extracellular elastolytic activity when grown on elastin-containing medium (Fig. 8). However, the relative activity was much less than that found in the extracellular fluid of elastin-grown A. fumigatus. One isolate (no. 28) produced exceptionally high levels of elastolytic activity compared with the other isolates of A. flavus (Fig. 8). This isolate was reconfirmed to be truly A. flavus and not A. fumigatus. Even this isolate produced much less elastase activity than that produced by the isolate no. 13 of A. fumigatus. To test whether A. flavus produces multiple forms of elastase and to compare the elastases produced by the two species of Aspergillus, the culture fluid of elastin-grown fungus was subjected to FPLC under the same conditions as used for A. fumigatus. Elastolytic activity eluted as one peak that corresponded to a molecular mass of 32 kDa in this gel filtration system (Fig. 5).

The apparent size was clearly larger than that produced by A. fumigatus. To test whether the elastase from A. flavus is immunologically related to that from A. fumigatus, a Western blot was performed on the crude culture fluid of A. flavus with polyclonal rabbit antiserum prepared against the purified elastolytic protease from A. fumigatus. A doublet of bands showing cross-reactivity was observed at 34 and 36 kDa (Fig. 9). This result suggests that the elastolytic protease from A. flavus is immunologically related to that produced by A. fumigatus.

Elastolytic enzyme from A. flavus that was first thought to be an SH protease was more recently reported to be a metalloprotease (31), whereas our results show that the
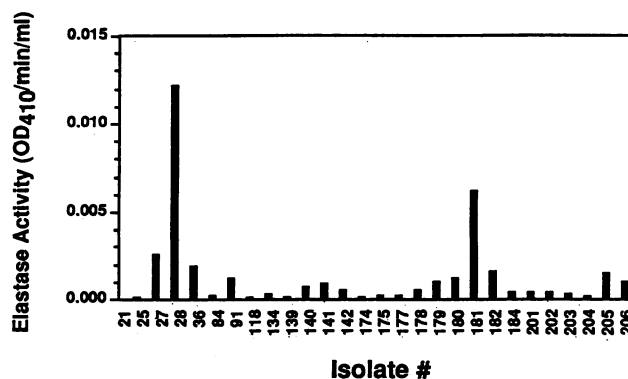
FIG. 8. Elastolytic protease production by hospital isolates of A. flavus grown on elastin-containing liquid medium. Activity levels were measured after 5 days of growth with N-succinyl-Ala-Ala-Pro-Leu-p-nitroanilide. (y-axis: Elastase Activity (OD_{410}/min/ml), 0.000–0.015; x-axis: Isolate #, isolates 21, 25, 27, 28, 36, 64, 84, 91, 118, 134, 139, 140, 141, 142, 174, 175, 177, 178, 179, 180, 181, 182, 184, 201, 202, 203, 204, 205, 206.)
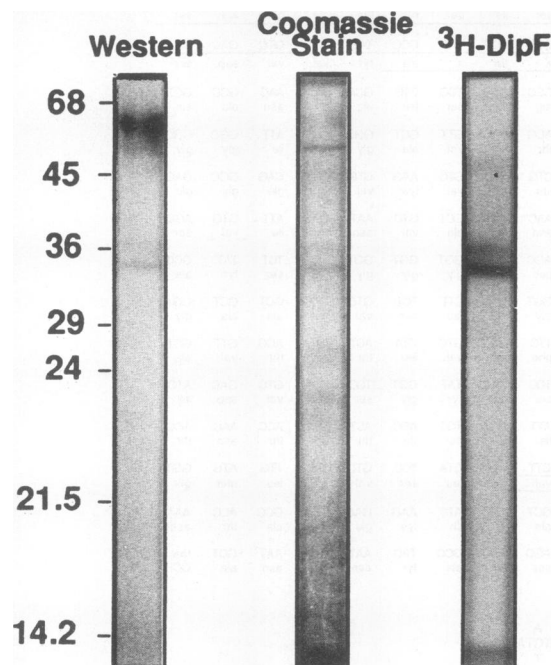
FIG. 9. SDS-PAGE of elastolytic protease in the extracellular fluid from *A. flavus* isolate no. 28 grown in elastin-containing medium. The Western blot was done with rabbit antibodies prepared against the purified elastolytic protease from *A. fumigatus* and ^{125}I-labeled protein A. The total protein from the extracellular fluid was treated with [^{3}H]DipF and subjected to SDS-PAGE followed by autoradiography. The middle panel shows the Coomassie blue-stained proteins in the extracellular fluid.

elastolytic enzyme produced by *A. fumigatus* is a serine protease. It would seem unusual for elastolytic enzymes from two species of *Aspergillus* to use distinctly different catalytic mechanisms. Therefore, we tested the elastolytic enzyme produced by *A. flavus* isolate no. 28 for the involvement of the active serine catalytic triad. Treatment of the enzyme preparation from *A. flavus* with increasing concentrations of DipF caused progressively higher inhibition of elastolytic activity, reaching a maximum of more than 80% inhibition at 1.0 mM DipF (Fig. 3). This inhibition was similar with either the peptide or [^{3}H]elastin as substrate. Even though the enzyme from *A. flavus* might be slightly less sensitive to DipF than that from *A. fumigatus*, the results strongly suggest that the elastolytic protease from *A. flavus* is a serine protease. In support of this conclusion, the histidine-directed reagent, DEPC, inactivated the *A. flavus* elastolytic enzyme in a concentration-dependent manner. This enzyme was at least as sensitive to inhibition by DEPC as was the elastolytic enzyme from *A. fumigatus*. This result strongly suggests the involvement of histidine, another member of the active serine catalytic triad, in the elastolytic activity of the enzyme. Treatment of the culture fluid of *A. flavus* with [^{3}H]DipF followed by SDS-PAGE and autoradiography showed a doublet of bands at 34 and 36 kDa (Fig. 9). These bands correspond to those which cross-reacted with the antibodies against the elastolytic enzyme from *A. fumigatus* and a doublet of Coomassie blue-staining bands (Fig. 9). Thus, the elastolytic enzyme produced by *A. flavus* uses active serine catalytic triad and is immunologically related to the enzyme from *A. fumigatus* although the two

might differ slightly in size. The apparent size difference is probably not due to differences in carbohydrate content because the enzyme from neither source showed any indication for the presence of carbohydrates even when a highly sensitive Glycotrack method (Oxford GlycoSystems) was used (data not shown).

Immunological detection of fungal elastolytic enzyme production in the host. To determine whether *A. fumigatus* secretes the elastolytic enzyme during the infection of the lungs of the host, an immunogold method was used. A preliminary cytochemical examination indicated that in the murine lungs inoculated by our method, conidia germinated in about 6 to 10 h. On the basis of these results, immunogold labeling was done on lung tissue of neutropenic mice 4 to 24 h after inoculation with conidia of *A. fumigatus*. Specific gold labeling was observed when the antiserum prepared against the elastolytic enzyme from *A. fumigatus* was used, but no labeling was observed with preimmune serum (Fig. 10). Spores germinating in the lung were found to be producing elastase that appeared to be secreted as the gold labeling was in the cell walls that appeared translucent; little gold labeling was found within the fungal cell. The secretion appeared to be targeted towards the germination point although not found exclusively at that point (Fig. 10A). Similarly, when germinated conidia appeared to be penetrating the host, the elastolytic enzyme appeared to be secreted with some apparent targeting towards the advancing growing point (Fig. 10B). These results are consistent with the hypothesis that *A. fumigatus* uses secreted elastolytic protease to degrade structural elastin in the host to help invade the host tissue.

Decreased virulence of an elastase-deficient mutant. If degradation of the structural barrier of the host by the fungal extracellular elastolytic enzyme is necessary for infection, this protease would be an important virulence factor. To test this possibility, a mutant of *A. fumigatus* isolate no. 13 deficient in the elastolytic enzyme was selected by screening mutants produced by chemical mutagenesis for production of elastolytic activity. The several mutants that showed lack of clearing on elastin plates were examined for their ability to produce elastolytic enzyme when grown on elastin medium. One mutant produced <10% of the elastolytic activity produced by the parent isolate (no. 13). The virulence of this mutant was compared with that of the wild type in a murine model (Fig. 11). Mice became neutropenic 3 days after irradiation with 400 rads. At this time, they were inoculated intranasally with conidia of *A. fumigatus* isolate no. 13 or its mutant deficient in elastolytic activity. Control mice that received no irradiation were also inoculated with conidia; irradiated control mice received saline containing no conidia. Both control groups showed no mortality. On the other hand, 9 out of 14 mice inoculated with the wild-type *A. fumigatus* died within 6 days but only 3 of 14 inoculated with the same number of conidia of the mutant died during this period. The experiment was repeated with similar results; the wild type and mutant caused the death of 8 and 3 mice, respectively, out of 14 mice in each group. Thus, elastolytic activity appears to be an important virulence factor.

DISCUSSION

The results presented in this paper strongly support the hypothesis that extracellular elastolytic activity produced by *A. fumigatus* is one of the important virulence factors involved in invasive aspergillosis. A general correlation of elastase production by some strains of *A. fumigatus* with
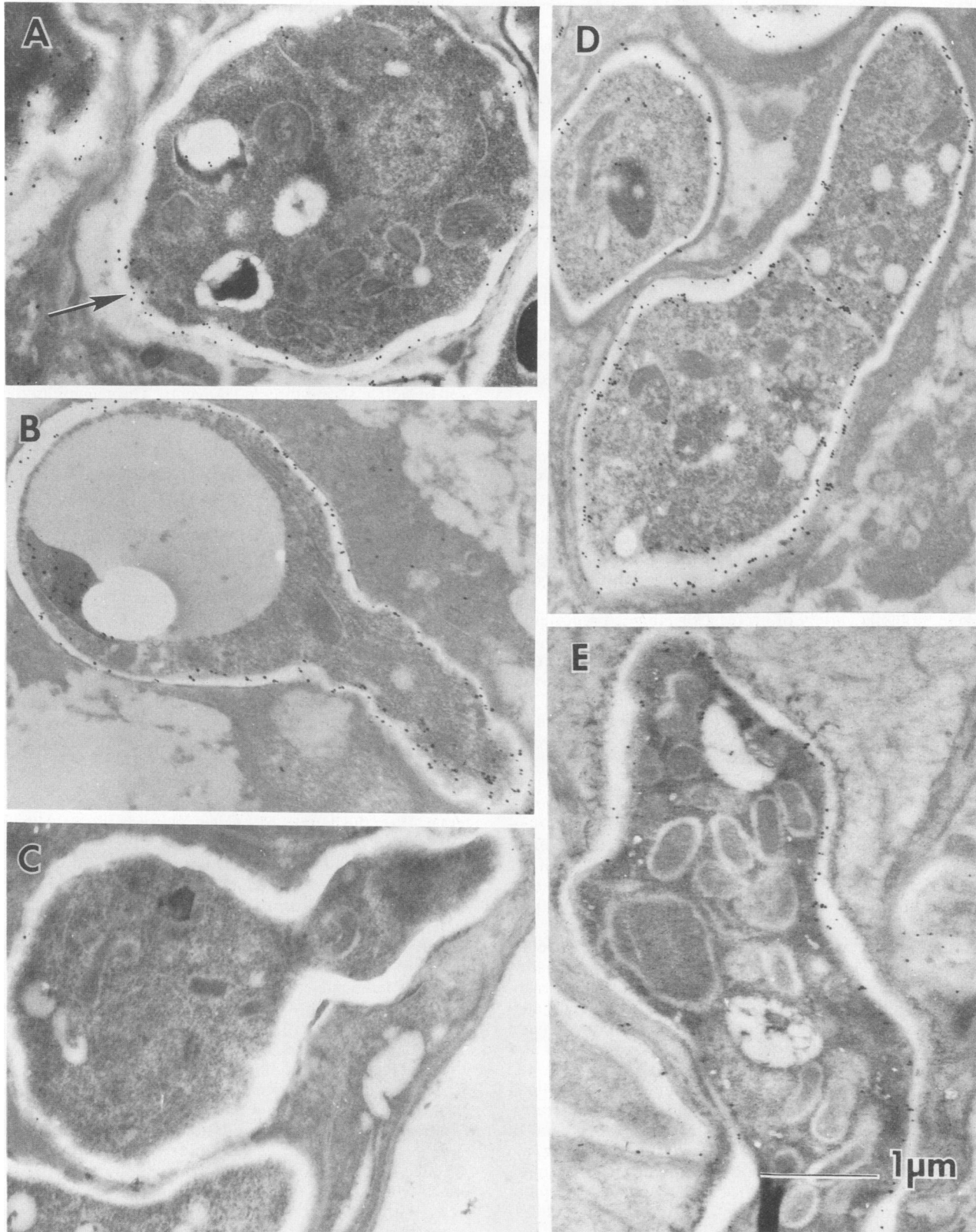
FIG. 10. Immunogold localization of elastolytic protease secreted by *A. fumigatus* in the host (mouse) lung. Mice were nasally inoculated with *A. fumigatus* conidia as described in the text, and after various periods of time lung sections were incubated with antiserum or preimmune serum, labeled with colloidal gold, and examined by electron microscopy. (A) 8 h after inoculation when germination was beginning; (B and D) 24 h after inoculation when hyphae were penetrating lung tissue; (C and E) 16 h after inoculation; (C) control in which preimmune serum was used.
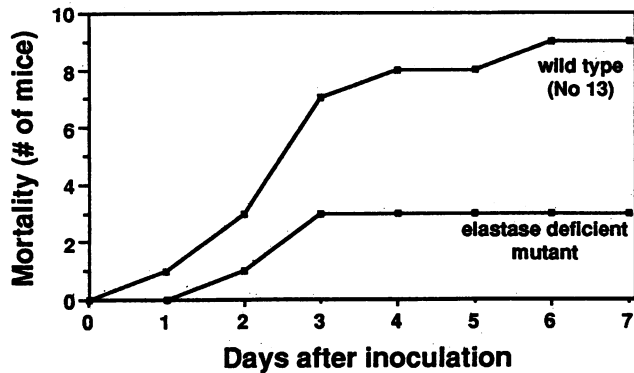
FIG. 11. Mortality of neutropenic mice inoculated with wild type and an elastase-deficient mutant. Fourteen mice were used for each treatment. Experimental details are given in Materials and Methods. By 7 days, recovery from neutropenia began and the conidia began to be cleared.

ability to cause pulmonary invasive aspergillosis in mice was reported (20, 37). The wide variation in the ability to produce elastolytic enzyme(s) that we observed is consistent with previous reports. If elastolytic enzyme is an important virulence factor, our finding that *A. flavus* isolates produce much less elastolytic activity than the *A. fumigatus* isolates is consistent with the finding that *A. fumigatus* accounts for the largest number of pathologic states involving *Aspergillus* species (5).

Even though a variety of proteases have been reported to be produced by *Aspergillus* species (2, 17, 23, 25, 27, 40), our results indicate that *A. fumigatus* and *A. flavus* produce only one extracellular elastolytic protease when grown on elastin. Alkaline proteases that showed a broad pH optimum of 7 to 10 and proteases with optimal activity at around pH 3.0 have been found. An extracellular protease from *A. fumigatus* was found to hydrolyze polyglutamic acid at pH 4.0 and polylysine at pH 10.0, and in both cases the enzyme produced peptides but not monomer (23). More recently, elastin-hydrolyzing enzymes have been isolated from *A. fumigatus* and *A. flavus* (11, 26, 29, 31). The extracellular serine protease recently isolated from a clinical isolate of *A. fumigatus* that was concluded to be similar or identical to the main chymotryptic activity of *A. fumigatus* showed properties similar to those of the present elastolytic protease (29) and a similar enzyme recently purified from another isolate of *A. fumigatus* (11). An alkaline protease-deficient transformant of *A. fumigatus* was recently reported to have drastically reduced elastase activity (39). This result would suggest that the alkaline protease that they characterized might be responsible for the elastolytic activity. The molecular size and the sequence similarity with the elastase that we purified suggest that these enzymes are quite similar if not identical. With other substrates such as casein and peptide substrates, no other protease was detected in the extracellular fluid of our cultures of *A. fumigatus* or *A. flavus*. Since the enzyme we purified appears to be the only one generated upon growth on elastin, this might be the only extracellular elastolytic protease produced by this fungus. The cell extracts would probably contain cellular proteases that are likely to include a variety of other proteases.

The elastolytic protease from *A. flavus* that was earlier considered to be a thiol protease was more recently reported to be a metalloenzyme (27). Our results strongly suggest that the elastolytic enzymes from both *A. fumigatus* and *A.*

flavus are serine proteases. Our conclusion that the enzyme from *A. fumigatus* is a serine protease, based on the results obtained with reagents that are known to selectively modify active serine and histidine, is strongly supported by its similarity to the enzymes reported by others (11, 26, 29) that are likely to be very similar to the enzyme we purified and characterized. This conclusion is strongly supported by the similarity between the amino acid sequence deduced from the PCR-generated cDNA for *A. fumigatus* enzyme that we present in this paper and that of an alkaline protease from another isolate of *A. fumigatus* (16). The present elastolytic enzyme from *A. fumigatus* showed the active-site motif G-T-S-M-A-T-P-H-I-V around the putative active serine. This sequence is more similar to that found in subtilisin than to that found in mammalian elastases.

We found that *A. flavus* when grown on elastin also produced one extracellular elastolytic protease; no other extracellular protease could be detected even with other substrates. This extracellular elastolytic enzyme was inhibited by active serine-directed reagents. The sensitivity of the *A. flavus* enzyme to DipF was quite similar to that of the *A. fumigatus* enzyme. Furthermore, the elastolytic proteases from the two sources showed identical sensitivity to DEPC, a reagent known to react selectively with histidine residues (histidine is a known component of the active serine catalytic triad). Treatment with radioactive DipF resulted in covalent attachment of the diisopropylphosphoryl group to the elastase as shown by electrophoresis and autoradiography, clearly showing covalent modification of active serine. Therefore, we conclude that the elastases from both sources are serine proteases.

The extracellular elastolytic enzymes from *A. fumigatus* and *A. flavus* showed other similarities. Rabbit antibodies prepared against *A. fumigatus* enzyme cross-reacted with the *A. flavus* enzyme. Western blot analysis showed that the same protein band(s) that showed covalent attachment of [³H]diisopropylphosphoryl group also showed cross-reactivity with the antibodies. The molecular size of the *A. flavus* enzyme is slightly larger than that of the *A. fumigatus* enzyme. SDS-PAGE showed that the former was 2 to 3 kDa larger than the latter. FPLC gel filtration on a calibrated column also showed similar differences in size. However, for reasons that are not clear, gel filtration showed lower molecular weight than that deduced from SDS-PAGE. From the cDNA sequencing results, it appears that the results obtained with SDS-PAGE are closer to the true molecular weight of the enzyme from *A. fumigatus*. The enzyme probably interacts with the gel filtration matrix, yielding apparent molecular weight values lower than the true molecular weight. *A. flavus* elastase previously purified was reported to be a 23-kDa protein (31). In this case, the molecular weight was determined by SDS-PAGE but is considerably smaller than the values we obtained. Since the authors concluded that their protein had undergone proteolysis, the smaller size might be the result of such proteolysis. Although we also detected a doublet as reported by Rhodes et al. (31), both of our bands represent considerably larger proteins than those observed by those authors. Both of our bands were labeled with [³H]DipF and showed cross-reactivity with antibodies prepared against the elastolytic enzyme from *A. fumigatus*. Since growth of *A. flavus* on elastin showed only one protease and this elastolytic enzyme was found to be similar in many ways to the elastase from *A. fumigatus*, we conclude that the enzyme we characterized is the elastolytic enzyme from *A. flavus*. It would appear that the previously reported enzyme could also be a serine

enzyme, as the inhibition by chelators required quite high concentrations (31). The possibility that the previously reported enzyme from *A. flavus* is a different protease cannot be ruled out, although it could be a proteolysis product derived from the native elastolytic enzyme.

Production of elastolytic enzyme during growth on elastin may not reflect what happens when the fungus grows in the lung. Other proteases that are required for pathogenesis may be generated under such conditions. Our findings that *A. fumigatus* when presented with the insoluble material from murine and bovine lung secretes one major protease that is indistinguishable from the elastolytic enzyme produced when the fungi are grown on elastin argue against such possibilities. Other proteases including collagenase could not be detected. Since elastin is the major structural component of lung tissue (38), these results are not surprising and support the hypothesis that extracellular elastase is a major virulence factor in aspergillosis.

To determine whether the extracellular elastolytic enzyme produced by *A. fumigatus* when grown in elastin medium is also produced by the fungus during actual infection of the host lung, we used an immunocytochemical approach. The immunogold labeling clearly showed that the conidium, germinating in the lung, secretes an elastolytic enzyme that immunologically cross-reacts with the enzyme produced when the fungus is grown in elastin medium. The appearance and wall localization of the gold particles observed with the present antielastolytic enzyme were quite similar to those observed with another antigen secreted by *A. fumigatus* (22). It appeared that secretion of the elastolytic protease may be targeted towards the germ tube and advancing hyphal tip. These observations demonstrate that *A. fumigatus* secretes elastolytic enzyme during infection of its host and support the hypothesis that the extracellular elastolytic enzyme helps this opportunistic pathogen to break down the major structural component of the host lung.

Correlation of elastolytic activity of *Aspergillus* isolates with their pathogenicity in immunocompromised animals suggested that elastolytic enzyme might be a virulence factor (5, 20, 37). This conclusion is supported by our recent experimental results in which chymotrypsin inhibitor from potatoes that inhibits the elastolytic enzyme from *A. fumigatus* as reported here also reduced mortality of mice caused by *A. fumigatus* (unpublished results). An elastase-deficient mutant of *A. flavus* generated by chemical mutagenesis was reported to be less virulent in immunocompromised animals (30). Our results provide further support for the hypothesis that extracellular elastolytic enzyme is a virulence factor that contributes significantly to mortality. The *A. fumigatus* mutant that generated less than 10% of the elastolytic activity of the wild type showed drastically reduced mortality in immunocompromised mice. In our murine model, 10% of the total number of conidia administered reached the lung and 10^7 to 10^9 conidia per mouse resulted in significant to almost complete mortality. Mortality varied with the age of the conidia; older, darker conidia caused higher mortality than younger, light-colored conidia. Therefore, we always used conidia from 10- to 14-day-old cultures that were dark colored. In spite of some variation, repeated experiments showed obviously lower mortality with the elastase-deficient mutant. Lack of mortality by a gene-disrupted transformant could yield a more convincing proof for the involvement of the elastolytic protease in aspergillosis if other compensatory increases do not occur in the expression of other genes that encode elastolytic proteases. Research to test these possibilities is in progress.

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