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Correlation of Elastase Production by Some Strains of *Aspergillus fumigatus* with Ability to Cause Pulmonary Invasive Aspergillosis in Mice†

MAHENDRA H. KOTHARY, THEODORE CHASE, Jr., AND JAMES D. MACMILLAN*

Department of Biochemistry and Microbiology, New Jersey Agricultural Experiment Station, Cook College—Rutgers University, New Brunswick, New Jersey 08903

Received 13 May 1983/Accepted 18 October 1983

Seventy-five strains of *Aspergillus fumigatus* were screened for production of elastase in liquid and agar media containing elastin in yeast carbon base buffered with 0.05 M borate, pH 7.6. Of 71 strains which cleared elastin in agar plates, 33 produced elastase in liquid medium, as measured spectrophotometrically with elastin-Congo red. Six strains producing elastase and four nonproducers were tested for ability to cause invasive aspergillosis in immunocompromised mice (six mice per strain). All 36 mice exposed to elastase-producing strains died within 48 to 96 h. Lung tissue from dead mice showed hyphae and necrosis of the alveoli. Lungs of mice exposed to spores of strains not producing elastase showed few germinated spores and no destruction of alveoli. These results indicate that elastase may be significant in the invasion process.

Aspergillus fumigatus is the usual etiological agent of aspergillosis in humans. Although in 1978 only 98 deaths were attributed to aspergillosis (5), the disease has gained significance because of its frequent occurrence in patients receiving cytotoxins, steroids, and organ transplants. Consequently, this disease of medical progress has stimulated interest in determining the mechanism of pathogenicity of *A. fumigatus*.

Sidransky et al. (22) and Gefter et al. (6) suggested that proteolytic enzymes enable aspergilli to invade lung tissues. It is increasingly apparent that elastases play a direct pathogenic role in various diseases of organs and tissues that contain elastin. There is evidence that elastases are responsible for lung lesions associated with emphysema (10, 11). Elastase involvement is well demonstrated for infections caused by *Pseudomonas aeruginosa* (2, 4, 27). Elastases produced by *Staphylococcus epidermidis* (15, 25), *Bacteroides nodosus* (23), and some dermatophytes (18, 19, 26) are also implicated in disease processes. Some investigators (9, 14) have speculated that proteases of *A. fumigatus* have a pathogenic function. It seemed possible to us that in pulmonary aspergillosis an elastase is involved in damage and destruction of lung tissue. However, there are no studies showing that *A. fumigatus* produces elastase.

In an earlier study (submitted for publication), we reported on 75 strains of *A. fumigatus* from air samples obtained in the vicinity of a sewage sludge composting site. In the present work, we screened these isolates for production of extracellular elastase by using a defined medium with elastin as the only nitrogen source. Although amounts varied, most cultures produced detectable amounts of the enzyme. Additional studies with immunocompromised mice as model system indicated the strong possibility that elastase does indeed play a role in the invasion of lung tissue by *A. fumigatus*.

* Corresponding author.

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MATERIALS AND METHODS

A. fumigatus. Cultures were isolated as previously described (12) from plates, incubated at 50°C, containing oxgall antibiotic agar (13) and exposed in an air sampler at a sewage sludge composting site located in Camden, N.J. Seventy-five isolates, identified as *A. fumigatus* by cultural and morphological characteristics (17), were selected for this study. The isolates were collected over the course of a year under a wide range of climatic conditions, and they included strains exhibiting a variety of morphological and colonial differences. Cultures were maintained on malt extract agar (Difco Laboratories, Detroit, Mich.) and stored under oil at 4°C.

Protease production in liquid media. Cells were grown either in liver medium (0.4% liver [Difco], 0.5% $CaCO_3$, 1.17% yeast carbon base [YCB; Difco]) or elastin medium (0.2% elastin [Sigma Chemical Co., St. Louis, Mo.], 0.2% YCB in 0.05 M borate buffer, pH 7.6). Sterile 0.1% Tween 80 was used to collect spores from a 3-day-old slant of *A. fumigatus* grown on malt extract agar. The spores were diluted to 10^7/ml with 0.1% Tween 80. One milliliter was inoculated into 24 ml of growth medium in a 250-ml baffled flask and was incubated on a shaker (150 rpm) at 37°C for 72 h.

Elastase production on solid medium. The medium contained 0.05% elastin, 0.05% YCB, 0.01% rose bengal (Allied Chemical & Dye Corp., New York, N.Y.), and 1.5% agar (Difco) in 0.05 M borate buffer, pH 7.6. Plates containing 20 ml of solidified medium were inoculated in a central spot with a loopful of spore suspension (10^7/ml). The cultures were incubated at 37°C for 7 days, and elastase activity was observed as a zone of clearing around and beneath the colony. The diameter of the clearing zone for a given strain did not correlate with measurement of elastase activity for the same strain grown in liquid medium.

Measurement of enzymatic activities. Protease activity was assayed by a modification (9) of the procedure of Anson (1) using 1% casein (Difco) in carbonate-bicarbonate buffer, pH 10, as a substrate. The reaction mixture, consisting of 0.1 ml of culture broth and 0.5 ml of casein (previously boiled for 15 min at pH 10), was incubated at 37°C for 10 min. One milliliter of 0.3 M trichloroacetic acid was added, and the

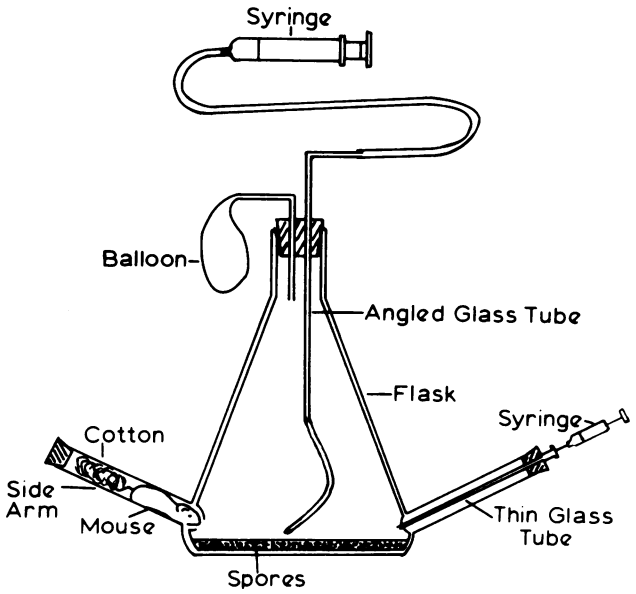

FIG. 1. Inhalation chamber for exposing mice to spores of *A. fumigatus*. Mice were inserted nose down into eight side arms. Spores from culture grown in the bottom of the flask were dispersed by injection of air through the angled tube. After exposure, air (sampled through a side arm) was injected into a diluent and plated for spore enumeration.

mixture was vortexed and then centrifuged at 1,000 × *g* for 10 min. One milliliter of 0.5 N NaOH was mixed with 0.5 ml of the supernatant liquid, and 0.3 ml of diluted (33%) phenol reagent (Folin-Ciocalteu; Fisher Scientific, Springfield, N.J.) was added. Absorbance was read at 660 nm. One unit of protease is that amount of enzyme which releases (in 10 min at 37°C) acid-soluble peptides yielding an absorbance of 1.0 at 660 nm.

Elastase was assayed with elastin-Congo red (Sigma) as a substrate (21). A standard curve was prepared by reacting different amounts of the substrate with porcine pancreatic elastase (Sigma) to complete hydrolysis and plotting the increase in absorbance against milligrams of elastin-Congo red. This curve was used to estimate amounts of Congo red released by *Aspergillus* elastase. One unit is that amount of enzyme releasing soluble dye equivalent to 1 mg of elastin-Congo red in 3 h at 37°C.

A plate diffusion assay (20) was modified and used for confirming elastase activity. Elastin (0.5 mg/ml) was suspended in 0.025 M borate buffer, pH 8.8, containing 1% purified agar (Difco). For the elastase assay, 100 μl of filter-sterilized culture broth was added to wells (diameter, 7 mm) in plates (15 by 100 mm) containing 20 ml of the agar mixture. After incubation at 37°C for 24 or 48 h, clearing zones developing around wells were observed as an indication of elastase activity. Standard curves were prepared by plotting the square of the clearing zone diameter against units of porcine pancreatic elastase. One unit of porcine pancreatic elastase solubilizes 1 mg of elastin in 20 min at pH 8.8 and 37°C. These standard curves were used to estimate *Aspergillus* elastase in terms of the porcine pancreatic elastase unit.

Infection model. A chamber (1-liter Erlenmeyer flask with eight side arms) similar to that described by Piggot and Emmons (16) was used to expose mice to inhalation of an aerosol of *A. fumigatus* spores (Fig. 1). Six strains which were strong producers of elastase and four nonproducing strains were selected for the infection studies. Sterile malt extract agar (200 ml) in the bottom of the chamber was inoculated with 10^7 spores. After incubation for 5 days at 37°C, a thick layer of the sporulating fungus completely covered the agar surface.

White male mice (CF1) weighing 18 to 20 g were obtained from Charles River Breeding Laboratories, Inc., Kingston, N.Y. Mice were immunocompromised with 2 mg of cortisone acetate (Sigma) in 0.2 ml of 0.1% Tween 80, injected subcutaneously 2 days before the mice were made to inhale spores. These mice also received 50 μg of tetracycline hydrochloride (Sigma) per ml in their drinking water. Seven immunocompromised mice and one untreated control mouse were exposed to spores in each experiment. In addition, a treated and an untreated mouse which were not exposed to spores were used as controls.

Mice were introduced into the side arms of the chamber, and spores were dispersed from the culture below by pumping 100 ml of air (in about 15 s) through an angled tube (Fig. 1), rotated once during pumping to ensure that spores were evenly distributed into the air in the upper part of the chamber. Mice inhaled spores for 3 to 4 min. At the end of the exposure period, a thin glass tube connected to a syringe was introduced into a side arm, and 4 ml of air was withdrawn from the chamber. The air sample was injected into 4 ml of 0.1% Tween 80 in a serum-stoppered vial. The

TABLE 1. Production of protease and elastase by selected strains of *A. fumigatus*

Strain no.	Enzyme production (U/ml)					Growth on solid elastin medium[c]
	Liver medium		Elastin medium			
	Protease	Elastase[a]	Protease	Elastase		
				Test A[a]	Test B[b]	
18	14	0	3.5	2.9	0.8	+ (25)
35	15	0	2.7	2.4	0.5	+ (31)
44	6	0	2.0	2.3	0.5	+ (52)
58	15	0	2.4	2.7	0.8	+ (28)
2	14	0	0.05	0.14	0	−
8	14	0	0.11	0.06	0	−
10	10	0	0.13	0.02	0	−
31	9	0	0.07	0.08	0	−

[a] Elastin-Congo red assay of culture broth.
[b] Plate diffusion assay of culture broth.
[c] Values in parentheses with positive tests show diameters (in millimeters) of clearing zones.

TABLE 2. Effect of YCB on clearing of 0.05% elastin by *A. fumigatus* 18.

YCB (%)	Clearing zone diam (mm)[a] on day:		
	3	4	5
0.01	0	0	33
0.02	0	0	32
0.04	0	18	38
0.05	20	28	40
0.1	0	26	39
0.2	0	0	35
0.4	0	0	0
0.8	0	0	0

[a] Clearing zones were usually smaller than the diameter of the colony and were measured from the underside of the plate.

number of viable spores was determined by plating dilutions onto Sabouraud dextrose agar (BBL Microbiology Systems, Cockeysville, Md.) containing 1.5% oxgall and then incubating the plates at 37°C for 48 h.

The approximate number of spores retained by the mice was determined immediately after exposure by plating serial dilutions of a homogenate of the lungs from one immunocompromised mouse on Sabouraud dextrose agar.

Mice were observed daily until death or for a period up to 4 weeks. Dead mice were necropsied, and their lungs were removed. The number of CFU in the left lung was determined by homogenizing and plating on Sabouraud dextrose agar. The right lung was fixed in 10% Formalin. Some sections were stained with hematoxylin and eosin or by the Gomori methanamine silver nitrate technique (7) for observation of fungi.

RESULTS AND DISCUSSION

Elastase and protease activity. All 75 strains tested exhibited protease activity in the liver medium. Seventy-one strains showed elastase activity in liquid or solid elastin medium or both. No strains had elastase activity in liver medium, indicating that elastase may be an inducible enzyme.

Four strong producers of elastase and four nonproducers were compared for enzymatic activities (Table 1). In experiments with liver medium, Jönsson and Martin (9) demonstrated that a strain of *A. fumigatus* isolated from a case of human aspergillosis produced more protease activity than other strains from nonclinical sources. On liver medium, we found little significant difference in the amounts produced by the eight strains tested.

When grown in elastin medium, strains 18, 35, 44, and 58 exhibited relatively high elastase activities, as measured by both the elastin-Congo red and the plate diffusion assay systems. These strains were also capable of growth on solid elastin medium and produced visible clearing zones around and beneath the colonies. Frequently, the clearing zone did not extend beyond the periphery of the colony. The other four strains did not grow on the defined medium containing elastin, and culture broth did not contain elastase as measured by the plate diffusion assay. A small apparent elastase activity was detected for these strains by the elastin-Congo red assay, but this may have been an anomaly due to proteolytic cleavage of a contaminating (nonelastin) protein also complexed to Congo red. Shotton (21) suggests that elastin-Congo red may not be a useful substrate for the assay of crude elastase containing other proteolytic enzymes. Initial elastase attack could nick elastin and open up susceptible sites for subsequent proteolytic (nonelastase) release of Congo red. The facts that strains 2, 8, 10, and 31 did not grow on solid elastin medium or exhibit elastase activity by the plate diffusion assay are more reliable reasons for concluding that these cultures do not produce elastase.

When grown in elastin medium, strains 18, 35, 44, and 58 exhibited relatively high protease activities. These protease activities may be due to elastase alone or to other proteolytic enzymes. Very low protease activities were detected for the other four strains. Proteases may have been produced either constitutively or in response to contaminating proteins in the elastin (e.g., breakdown products of elastin owing to autoclaving).

The plate diffusion assay of Schumacher and Schill (20) required modification for our use because crude *Aspergillus* elastase exhibited considerably higher activity in 0.025 M borate buffer, pH 8.8, than in the 0.2 M (or 0.025 M) Tris-hydrochloride buffer, pH 8.8, employed in the original method. We did not use *Aspergillus* elastase to standardize the modified assay because the amounts of activity produced in culture were low and rather indistinct clearing zones were frequently produced, even after 48-h reaction times. A commercial source of porcine pancreatic elastase gave good linearity at 24 h and fair linearity at 48 h. These curves were used to estimate *Aspergillus* elastase activity in terms of porcine pancreatic elastase units.

The solid elastin medium, developed originally to deter-

TABLE 3. Mortality of immunocompromised mice (six for each strain) exposed to spores of elastase-producing and nonproducing strains of *A. fumigatus*

Strain no.	Elastase	Spore count		No. of surviving mice on day:				Mortality (%)	*A. fumigatus* in dead mouse lung (CFU $\times 10^3$)[a]
		Chamber ($\times 10^4$/ml)	Lungs ($\times 10^8$)	1	2	3	4–28		
18	+	1.3	2	6	3	0	0	100	200
35	+	2.6	5	6	2	0	0	100	800
44	+	1.1	2	6	2	0	0	100	700
58	+	6	7	6	3	0	0	100	80
19	+	2.1	6	6	1	0	0	100	ND[b]
34	+	1.0	2	6	3	1	0	100	ND
2	−	1.6	6	6	4	4	4	33	0.2
8	−	1.3	2	6	4	4	4	33	0.4
10	−	1.4	3	6	4	4	4	33	0.8
31	−	1.2	5	6	4	3	3	50	2

[a] Values refer to the total CFU detected in the left lung of one mouse tested from each group. No CFU were detected in the lungs of surviving mice.

[b] ND, Not determined.

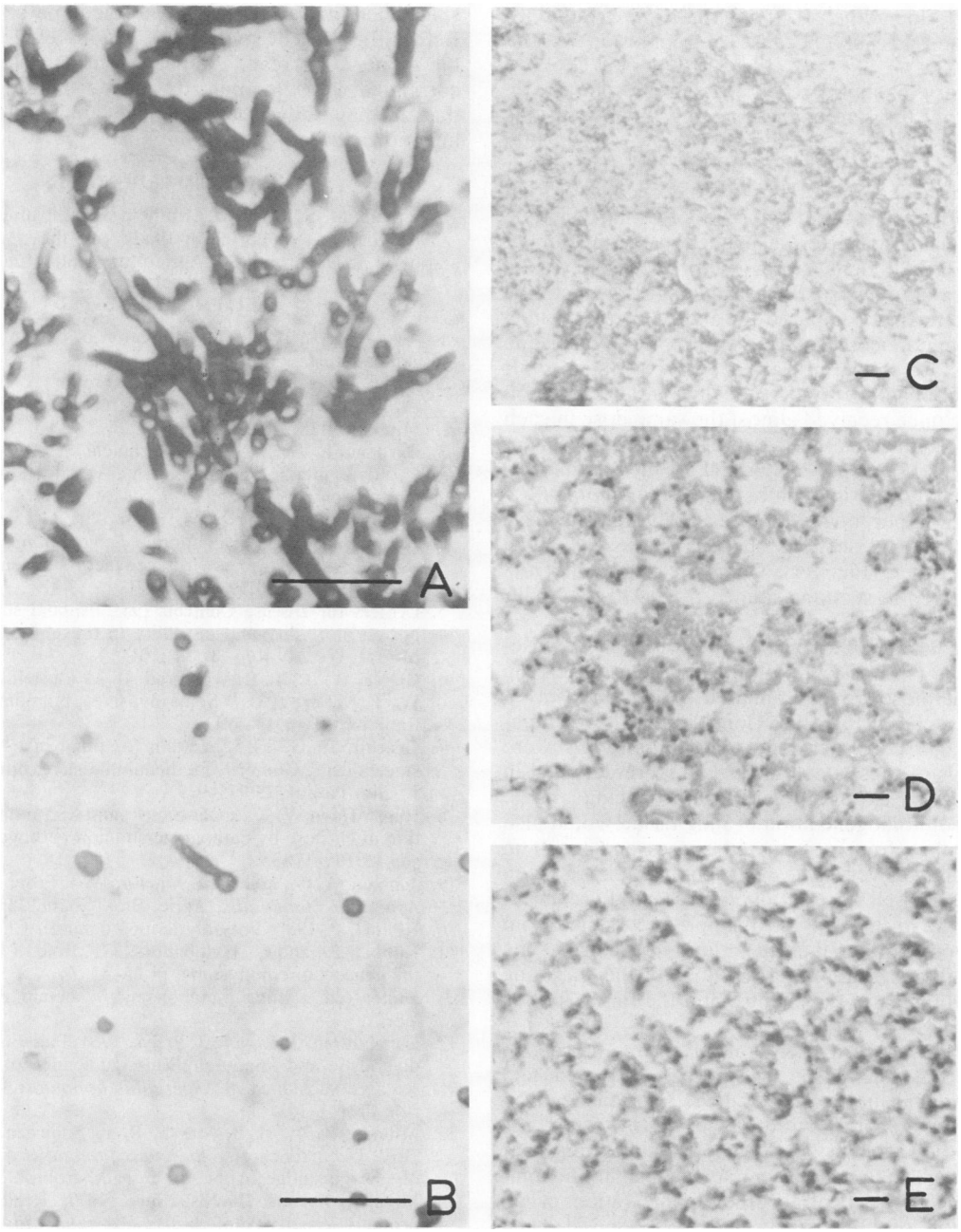

FIG. 2. Bright-field photomicrographs of mouse lung sections stained for fungi (A, B) or stained with hematoxylin and eosin (C, D, E). Tissue from a mouse killed by exposure to elastase-producing *A. fumigatus* shows hyphae (A) and necrosis of alveoli (C). Tissue from a mouse which died after exposure to non-elastase-producing *A. fumigatus* shows mainly ungerminated spores (B) and intact alveoli (D) comparable to those (E) from a mouse that survived exposure to the same strain. Bars, 20 μm.

mine whether *A. fumigatus* could use elastin for growth, was also very useful in detecting elastase activity of many strains which did not produce detectable amounts of the enzyme in culture broth.

Studies with strain 18 revealed that the amount of YCB incorporated into solid elastin medium had a significant effect on the clearing of elastin (Table 2). Maximum clearing was observed in a medium containing 0.05% YCB. Although there was good growth of the fungus (i.e., colonies were about 60 mm in diameter by day 5) on 0.4 and 0.8% YCB, no clearing of elastin occurred, even after 7 days. This lack of clearing may be due to repression of elastase production by the relatively high amounts of amino acids in 0.4 and 0.8% YCB compared with those in the lower concentrations employed. Significantly, non-elastase producers also grew on 0.4 and 0.8%, but not on 0.05%, YCB. With elastase producers, there was no clearing of elastin when peptone was added to the medium (results not shown). Evidently, the fungus does not produce elastase when other sources of nitrogen are available for growth. Hopsu-Havu et al. (8) employed peptone in the medium used to screen a large group of fungi and actinomycetes for elastase. None of the

aspergilli tested, including *A. fumigatus*, were found to produce elastase, possibly because of repression by the peptone.

It was necessary to employ rose bengal in the solid elastin medium to restrict the size of the colonies. Even with this dye, the diameter of the elastin clearing zone was frequently smaller than the colony diameter. It could be that higher amounts of elastase are excreted by older cells or that peripheral growth of cells occurs more rapidly than complete degradation of elastin particles suspended in the medium.

Model infection. All eight strains used for the enzymatic studies and two additional elastase-producing strains (strains 19 and 34) were further compared for ability to invade lung tissue in mice immunocompromised with 2 mg of cortisone acetate. Results are shown in Table 3. Spore counts of air from the exposure chamber and of the lungs after inhalation indicate that the mice received almost the same dose in each experiment. All 36 mice exposed to the six elastase-producing strains (strains 18, 19, 34, 35, 44, and 58) died within 2 to 4 days after inhalation. High numbers of *A. fumigatus* (8×10^4 to 8×10^5 CFU per lung) were present in lungs of these dead mice, and stained sections showed extensive hyphal invasion of the tissue (Fig. 2A). Complete necrosis of the tissue was observed in sections stained by the hematoxylin and eosin method (Fig. 2C).

Only 9 of the 24 mice exposed to the four non-elastase-producing strains (strains 2, 8, 10, and 31) died. The lung tissue contained mostly nongerminated spores and only a few germinated spores (Fig. 2B). Considerably lower numbers (200 to 2,000 CFU per lung) of *A. fumigatus* were detected in lungs of these dead mice than were detected in dead mice previously exposed to elastase-producing strains. In the latter case, homogenization of lung tissue would also homogenize hyphae from germinated spores and result in more CFU. There was very little destruction of lung tissue in mice exposed to non-elastase-producing strains (Fig. 2D). It is possible that the mice exposed to nonproducers died from an allergic response rather than from lung invasion. At the end of the 28-day observation period, none of the surviving mice had *A. fumigatus* in their lungs, and the tissue looked normal (Fig. 2E).

None of the 24 mice in three control groups (cortisone acetate-treated but unexposed to spores, untreated and unexposed, untreated but exposed) died. Lack of death in the last control group agrees with the fact that *A. fumigatus* is an opportunistic secondary pathogen. We have found that the amount of cortisone acetate employed to immunocompromise the mice is critical for the manifestation of an invasive infection of lung tissue. No mice died if 1-mg injections were used instead of 2 mg. With 5-mg injections, mice died even in the inhalation chamber, and all mice exposed to both elastase producers and nonproducers died within 12 h. Once again, lung tissue contained mainly nongerminated spores, indicating that death was due to some other factors, such as a severe allergic reaction.

Although we did not try different doses, it is likely that susceptibility to death by invasive infection could vary with the concentration of spores inside the lungs. If so, then it is also possible that the spore concentration and the amount of cortisone acetate employed for immunocompromisation are interdependent factors in the mouse model used to demonstrate invasive aspergillosis.

A. fumigatus is reported to produce endotoxins (24), C substance (12), fumitremorgins (28), and mycotoxins such as fumagillin, fumagatin, gliotoxin, and helvolic acid (3), which may be significant in disease processes. It has also been speculated that proteases play a role in pathogenesis (9, 14). Before the present study there was no evidence to support the theory. Our results indicate that elastase production is correlated with the ability to invade the lungs of mice and that it may indeed be a significant virulence factor in infections caused by *A. fumigatus*.

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LITERATURE CITED

1. **Anson, M. L.** 1938. The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. J. Gen. Physiol. **22**:79–89.
2. **Baker, N. R.** 1982. Role of exotoxin A and proteases of *Pseudomonas aeruginosa* in respiratory tract infections. Can. J. Microbiol. **28**:248–255.
3. **Bardana, E. J., Jr.** 1981. The clinical spectrum of aspergillosis. Part 1: epidemiology, pathogenicity, infection in animals, and immunology of *Aspergillus*. Crit. Rev. Clin. Lab. Sci. **13**:21–83.
4. **Blackwood, L. L., R. M. Stone, B. H. Iglewski, and J. E. Pennington.** 1983. Evaluation of *Pseudomonas aeruginosa* exotoxin A and elastase as virulence factors in acute lung infection. Infect. Immun. **39**:198–201.
5. **Centers for Disease Control.** 1981. Annual summary 1980: reported morbidity and mortality in the United States. Morbid. Mortal. Weekly Rep. **29**(54):101.
6. **Gefter, W. B., T. R. Weingrad, D. M. Epstein, R. H. Ochs, and W. T. Miller.** 1981. "Semi-invasive" pulmonary aspergillosis. Radiology **140**:313–321.
7. **Grocott, R. G.** 1955. A stain for fungi in tissue sections and smears using Gomori's methenamine-silver nitrate technic. Am. J. Clin. Pathol. **25**:975–979.
8. **Hopsu-Havu, V. K., C. E. Sonck, and E. Tunnela.** 1972. Production of elastase by pathogenic and non-pathogenic fungi. Mykosen **15**:105–110.
9. **Jönsson, A. G., and S. M. Martin.** 1964. Protease production by *Aspergillus fumigatus*. Agric. Biol. Chem. **28**:734–739.
10. **Kimbel, P.** 1980. Proteolytic lung damage. Chest **77**:274–275.
11. **Kimbel, P., and G. Weinbaum.** 1975. Role of leucoproteases in the genesis of emphysema, p. 25–41. *In* A. F. Junod and R. de Haller (ed.), Lung metabolism. Academic Press, Inc., New York.
12. **Longbottom, J. L., and J. Pepys.** 1964. Pulmonary aspergillosis: diagnostic and immunological significance of antigens and C-reactive substance in *Aspergillus fumigatus*. J. Pathol. Bacteriol. **88**:141–151.
13. **Millner, P. D., P. B. Marsh, R. B. Snowden, and J. F. Parr.** 1977. Occurrence of *Aspergillus fumigatus* during composting of sewage sludge. Appl. Environ. Microbiol. **34**:765–772.
14. **Miyaji, M., and K. Nishimura.** 1977. Relationship between proteolytic activity of *Aspergillus fumigatus* and the fungus' invasiveness of mouse brain. Mycopathologia **62**:161–166.
15. **Murphy, R. A.** 1974. Elastase production by oral staphylococci. J. Dent. Res. **53**:832–834.
16. **Piggot, W., and C. W. Emmons.** 1960. Device for inhalation exposure of animals to spores. Proc. Soc. Exp. Biol. Med. **103**:805–806.
17. **Raper, K. B., and D. I. Fennell.** 1965. The genus *Aspergillus*. The Williams & Wilkins Co., Baltimore.
18. **Rippon, J. W.** 1967. Elastase production by ringworm fungi. Science **157**:947.
19. **Rippon, J. W., and D. P. Varadi.** 1968. The elastases of pathogenic fungi and actinomycetes. J. Invest. Dermatol. **50**:54–58.
20. **Schumacher, G. F. B., and W. B. Schill.** 1972. Radial diffusion in gel for micro-determination of enzymes. II. Plasminogen activator, elastase and non-specific proteases. Anal. Biochem. **48**:9–26.
21. **Shotton, D. M.** 1970. Elastase. Methods Enzymol. **19**:113–140.
22. **Sidransky, H., S. M. Epstein, E. Verney, and C. Horowitz.** 1972.

Experimental visceral aspergillosis. Am. J. Pathol. **69**:55–67.
23. **Stewart, D. J.** 1979. The role of elastase in the differentiation of *Bacteroides nodosus* infections in sheep and cattle. Res. Vet. Sci. **27**:99–105.
24. **Tilden, E. B., E. H. Hatten, S. Freeman, W. M. Williamson, and V. L. Koenig.** 1961. Preparation and properties of the endotoxins of *Aspergillus fumigatus* and *A. flavus*. Mycopathologia **14**:325–346.
25. **Varadi, D. P., and A. C. Saqueton.** 1970. Perifollicular elastolysis. Br. J. Dermatol. **83**:143–150.
26. **Werb, Z., M. J. Banda, J. H. McKerrow, and R. A. Sandhaus.** 1982. Elastases and elastin degradation. J. Invest. Dermatol. **79**:154s–159s.
27. **Woods, D. E., S. J. Cryz, R. L. Friedman, and B. H. Iglewski.** 1982. Contribution of toxin A and elastase to virulence of *Pseudomonas aeruginosa* in chronic lung infections of rats. Infect. Immun. **36**:1223–1228.
28. **Yamazaki, M., S. Suzuki, and K. Miyaki.** 1971. Tremorgenic toxins from *Aspergillus fumigatus* Fres. Chem. Pharm. Bull. **19**:1739–1740.