Experimental Emphysema in Rats: Elastolytic Titer of Inducing Enzyme as Determinant of the Response[1] (37610)

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An association between emphysema and a rare inherited plasma protein abnormality, α_1-antitrypsin deficiency, was first described by Laurell and Eriksson in 1963 (1). Patients may develop the disease at a relatively early age if specific proteolytic enzymes more readily digest connective tissue elements of the lung. This theory has gained support when it was shown by Gross *et al.* (2) that experimental emphysema can be induced in animals by intratracheal injections of the proteolytic enzyme papain. Papain is one of the few proteinases not inhibited by α_1-antitrypsin, and this fact may account for its effectiveness at low doses. It is suggested that to inflict the damage which causes emphysema on living tissues, a critical titer must be exceeded. In man this threshold is lowered significantly in persons homozygously or heterozygously deficient in α_1-antitrypsin; in experimental animals it is assumed that the massive enzyme dose overcomes possible protective effects with concomitant morphological lesions in the lung parenchyma.

Although the extent of inhibitor deficiency is commonly measured by inhibitory capacity toward pancreatic trypsin, the causative agent must be sought elsewhere. Morphological examination of the damaged lung indicates that connective tissue proteins, unaffected by trypsin, are the primary target. We have suggested (3) that elastin damage precedes or accompanies functional impairment, and that an elastolytic enzyme, probably of leukocyte or macrophage origin, may be responsible for the *in vivo* destruction. Gross (4) has speculated that papain is more effective than trypsin because of its resistance to α_1-antitrypsin inhibition. This may be a factor but, in adddition, papain, unlike trypsin, has some elastolytic activity. Pancreatic elastase, which is inhibited by serum as strongly as trypsin, can, nevertheless, induce emphysema and our own experiments with thermolysin have shown a high degree of effectiveness with this microbial elastase (5). Additional support for the suggested role of elastolysis may be derived from our findings in 1969 (6) that serum from patients with emphysema associated with familial α_1-antitrypsin deficiency also lacked inhibitory capacity towards pancreatic elastase. More recently, Janoff (7) reported a similar deficiency of serum inhibitory capacity towards leukocyte elastase. The present study was undertaken to test the hypothesis that the effectiveness of an enzyme in the induction of experimental emphysema is directly related to its elastolytic titer.

Materials and Methods. For a more valid comparison, only microbial enzymes were included in this study. In addition to thermolysin, a *B. thermoproteolyticus* enzyme, which was received initially through the courtesy of Dr. S. Endo of Daiwa Kasei, Osaka, Japan and subsequently purchased from Calbiochem, we have used Brinase, an *Aspergillus oryzae* enzyme obtained through the courtesy of Astra, Sweden; Pronase, a *Streptomyces griseus* enzyme purchased from Calbiochem; an Actinomyces preparation, elastase ABC, obtained from Advance Biofactures Corp., Lynbrook, Long Island, and two preparations of *B. subtilis* enzymes, Bioprase NSP_4 from

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Nagase Co. and Takamine H. T. concentrate, Batch 4903, from the Miles Chemical Co., Elkhart, IN (formerly Takamine Laboratories, Clifton, NJ). The latter two enzymes were included because of the extensive use of similar enzymes in detergents and the reported effect of these enzymes on lung tissue in exposed factory workers as well as in hamsters. The batches used were assayed for elastolytic activity by a modified orcein–elastin method. Activity units are expressed as OD at 590 nm of orcein released from 20 mg substrate after 60 min incubation with 1 mg enzyme in 3 ml 0.2 *M* glycine buffer (pH 8.9) in a shaking waterbath at 37°. Total proteolytic activity was determined similarly with azocoll and expressed as OD per milligram enzyme incubated with 20 mg azocoll in 5 ml 0.2 *M* glycine buffer (pH 8.9) for 15 min in a shaking waterbath at 37°, centrifuged, and the supernatant color was read at 540 nm in a Bausch & Lomb spectrophotometer. Activities are listed in Table I together with relative toxicity determined by ip injection into female CF_1 mice. Toxicity is expressed as the reciprocal of the LD_{50} in milligrams of enzyme.

Aliquots of 0.5 and 1.0 mg of each enzyme dissolved in 0.9% NaCl were injected iv into the tail vein of Sprague-Dawley rats of 180–200 g weight. All rats were injected on Days 1, 4 and 7; half in each group were sacrificed by decapitation 1 wk after the last injection, the others received 4 additional injections on Days 12, 15, 19 and 22 and were sacrificed on Day 29. Lungs were inflated *in situ* with formalin and paraffin; sections 6 μm thick were cut and stained with H and E and with Verhoeff's elastin stain. Severity of emphysema was arbitrarily designated 1+ to 4+, based on the extent of elastin fragmentation, airspaces and club shaped ends, without prior knowledge of the treatment undergone by a given animal. Visual inspection of the stained lung section was also used to assess the degree of hemorrhage. Lack of toxicity was deduced from the steady weight gain of the animals.

Results. Figure 1 shows the average weight gain in each group after 1 mg injections were given up to 7 times at 3-day intervals. After 8 wk, the experimental animals, as well as the controls, had almost doubled the initial weight and no significant reduction in growth could be discovered. At the concentration used, none of the enzymes was excessively toxic. A relationship was discerned between elastolytic titer and experimental emphysema induction. The higher the elastolytic activity, the greater the severity of the lesion. The effect is a cumulative one, and higher doses or additional injections increase the likelihood of emphysema production or the severity of the lesions. This is brought out in Table II, showing that the *B. subtilis* enzymes with low elastolytic activity will not induce emphysema at concentrations found effective in the case of Actinomyces elastase; 3 injections of 1 mg were, however, more effective than 7 injections of 0.5 mg. Table II also compares the elastin fiber fragmentation and the incidence of hemorrhage. The total proteolytic activity determines the amount of hemorrhage. Thus pronase, with 1/10 the elastolytic activity of Brinase, was a poorer inducer of emphysema, but since it had twice the total proteolytic activity, it produced a similar degree of hemorrhage. Thermolysin, with only slightly less elastolytic activity than Brinase, was equally effective in induc-

TABLE I. Activity and Toxicity Titers of Experimental Enzymes.

Enzyme	Source	Elastase activity (orcein–elastin units/mg)	Total proteolytic activity (azocoll units/mg)	Toxicity (LD_{50} units/mg)
Brinase	*Aspergillus oryzae*	11.5	25	2.3
Thermolysin	*B. thermoproteolyticus*	8.5	213	2.61
Pronase	*Streptomyces griseus*	1.15	58	1.43
Elastase ABC	Actinomyces species	0.068	6.4	0.33
Takamine HT	*B. subtilis*	0.058	2.2	0.23
Nagase	*B. subtilis*	0.014	0.54	<0.02

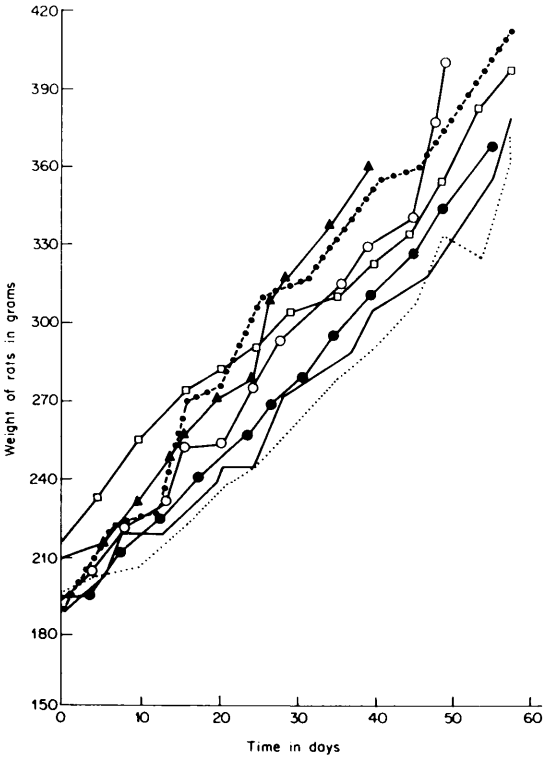

FIG. 1. Gain in weight of rats injected with 1 mg amounts of various proteolytic enzymes. (——) Control; (□—) brinase; (●-) thermolysin; (•—) pronase; (••••) elastase ABC; (○-) nagase; (▲—) takamine HT.

ing emphysema, but because its proteolytic titer was 8.5 times as high, it gave rise to a greater amount of hemorrhage. With any one enzyme the risk of hemorrhage depends on concentration and the number of injections, but under otherwise identical conditions, it is proportional to the proteolytic titer.

Discussion. The reported results indicate that the enzyme of choice for induction of emphysema in rats should have a high elastolytic to total proteolytic activity ratio. Of the enzymes included in this study, Brinase, with an activity factor of 1:2, comes closest to this prerequisite, surpassing thermolysin with a factor of 1:25. In pronase the factor is 1:50, similar to that of the *B. subtilis* enzymes which are, however, less effective because of their low absolute titers. Concomitant hemorrhage was only mild in our experiments in contrast to observations by Goldring, Ratner and Greenberg (8) who noted that administration of two *B. subtilis* proteases to Syrian hamsters, one intratracheally and one by aerosol, caused massive hemorrhage leading to death of 8 of 46 animals within the first 2 days. More recently, Goldring *et al.* (9) described additional effects of the detergent enzymes administered intratracheally. With Alcalase (Novo Industries, Copenhagen) one half the hamsters died within 24 hr when 9 mg/animal were injected, and one third when 4.5 mg were injected. With Maxatase (Pfizer) at higher pH, 6 out of 35 succumbed to 9 mg doses within 10 days. The greater toxicity may be due to associated impurities in the commercial enzyme preparations, to species differences or to high-

TABLE II. Dose Dependency of the Degree of Lung Damage Induced by Experimental Enzymes.

Enzyme	Amt injected (no. injections × mg)	Degree of emphysema	Hemorrhage (H&E, Verhoeff)	Elastin fiber breakage (Verhoeff)
Brinase	3 × 1.0	+++	+	++++
Thermolysin	3 × 0.5	+++	0	+++
	3 × 1.0	+++	++	++++
Pronase	3 × 0.5	++	0	++
	3 × 1.0	++	+	+++
Elastase ABC	3 × 0.5	0	0	0
	7 × 0.5	0	0	0
	3 × 1.0	+	0	+
Takamine HT	7 × 0.5	0	0	0
	7 × 1.0	+	0	+
Nagase	7 × 0.5	0	0	0
	7 × 1.0	+	0	+

er total activities. The authors did not determine proteolytic titer, but the morphological findings, as well as the nature of the enzymes, suggest that the proteolytic to elastolytic ratio was higher than in our experiments. Plant proteinases with elastolytic activity (papain, bromelin, bromelain, ficin, pinguianain) were found capable of inducing emphysema, but because of their relatively high nonspecific proteolytic activity, caused excessive hemorrhage. Trypsin, which has no elastolytic activity, was ineffective. Intravenous injections with crystalline pancreatic elastase consistently produced emphysema-like lesions in spite of its strong inhibition by circulating α_1-antitrypsin. Although the listed data refer to multiple injections, experimental emphysema can be induced by 2 injections of 2 mg or single injections of 5 mg pancreatic elastase, thermolysin or Brinase. These findings are borne out by two recent reports from other laboratories: Janoff (10) presented evidence that when emphysema was produced by intratracheal injections into Swiss-Wistar mice, thermolysin was more than 3 times as effective as pancreatic elastase and more than 20 times as effective as papain; Johanson and Pierce (11) found elastase superior to papain in the production of emphysema-like lesions by direct injection into isolated postmortem rat lungs, while collagenase had no effect.

The assumption that the primary target in human emphysema is indeed elastin has been strengthened by a recent investigation of lung connective tissue of penicillamine treated rats by Hofmann *et al.* (12). These authors concluded that abnormalities of the lung's compliance and elastic recoil, as in emphysema, were related to changes in the quality of the elastin. Association of histologically observed damage in the elastin, rather than the collagen moiety of lung connective tissue, with marked alterations in lung function and structure of rats following papain administration, has also been confirmed by Johanson *et al.* (11, 13). Our own chemical analyses of isolated lung elastin from human autopsy material derived from patients who died with emphysema (3), and from rats with thermolysin-induced experimental emphysema (5), revealed extensive deviations from normal in the respective amino acid compositions.

The results of the present study support our initial hypothesis; they indicate a rational basis for inducing experimental emphysema without excessive hemorrhage and, more important, they throw light upon the probable mode of emphysema production in man. In accord with other evidence, any factor tending to promote hydrolysis of lung elastin would increase susceptibility to environmental or other results by lowering the threshold for tissue destruction. Deficiency in α_1-antitrypsin would have this effect, but so would alterations in the molecular architecture of the elastin molecule or local increases in concentration of elastase releasing leukocytes. Experiments designed to assess the relative contributions of these and other possible mediators of pulmonary injury are in progress.

Summary. Emphysema-like lesions have been produced in rat lungs by iv administration of various proteolytic enzymes. The severity of the reaction was found to depend on the activity of the enzyme injected. The effect of six microbial proteases was compared in terms of ease of emphysema induction, degree of damage of the fiber structure and accompanying hemorrhage. A positive relationship was established between elastolytic titer of the inducing enzyme and development of emphysematous lesions. On the other hand, hemorrhage was proportional to total proteolytic titer. Best results were obtained when the ratio of specific elastolytic to total proteolytic activity was high.

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