PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 146, 712–717 (1974)

In Vivo Effects of Pancreatic Elastase I—Studies on the Serum Inhibitors[1,2] (38178)

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(Introduced by L. J. Cizek)

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Several pathological processes in man and animals involve the alteration or destruction of elastic fibers. Such processes include arteriosclerosis and pulmonary emphysema (1–3). The destruction of elastic fibers in such diseases is often attributed to elastolytic enzymes such as pancreatic elastase (1), or the elastase present in leukocytes (4) and blood platelets (5). Pancreatic elastase *per se* has been suggested (6–8) as a cause of tissue lesions although no evidence has been produced in favor of its passage from the pancreas into the circulation. It has also been reported that several serum protease inhibitors which are active against trypsin and chymotrypsin do inhibit pancreatic elastase (9, 10) and to a less extent leukocyte elastase (4).

The absence or an abnormally low level of such inhibitors could be considered as a factor in the alteration of the elastic fibers in lung and elsewhere by circulating elastolytic enzymes (3, 11, 12).

The availability of pancreatic elastase with a known amino acid sequence and tertiary sequence in a crystalline, highly purified form (13, 14) allows such a hypothesis to be tested experimentally *in vivo*. In this study, experiments were carried out to investigate the tissue alterations produced by intravenously injected pancreatic elastase and its effect on the levels of serum protease inhibitors. From such experiments indirect conclusions may be drawn regarding the *in vivo* interaction between serum inhibitor concentrations and low and high concentrations of circulating elastolytic enzymes.

Materials and Methods. To provide an animal preparation which would allow adequate blood sampling and suitability for physiological testing while conserving the amounts of enzymes required for an effect, puppies (beagle strain) with a weight range of 3 to 5.5 kg were selected.

Each animal received a single intravenous elastase injection of varying concentration. Blood was sampled for the analysis of protease inhibition before injection and at intervals after injection by separate venapuncture at intervals ranging from 30 min to 24 hr. Crystallized porcine pancreatic elastase (EC 3447) was obtained from Whatman Biochemicals, Springfield Mills, Maidstone, Kent, England. It was dissolved in sterile 0.9% NaCl for injection, at a concentration of 20 to 80 mg/ml.

The total dose of elastase injected in each animal ranged from 20 to 200 mg.

[1] Work done while a career investigator of the Health Research Council of the City of New York under contract I 182.

[2] Work supported by grants from the POLACHEK FOUNDATION and by the Health Research Council of the City of New York. Contract #U2237 and U.S.P.H.S.H.L.15832 also supported by D.G.R.S.T. (Action concertee "Echanges Respiratoires et Circulatoires" and Action Complementaire Coordonnee "Biologie et Pathologie des Parois Arterielles et Arteriolo-capillaires"), by C.E.A. and by C.N.R.S., and by I.N.S.E.R.M. Contract No. 714054.

Control experiments were carried out by injecting puppies intravenously with equal volumes of sterile saline alone.

Serum was separated from the blood samples and kept frozen.

Three times crystallized porcine trypsin was obtained from Worthington Biochemicals, Freehold, New Jersey.

Determination of serum elastase inhibitor was carried out by using ^{125}I-labelled elastin as substrate, as described (15). Elastin was obtained from pig aorta using the NaOH purification procedure (16). Serum trypsin inhibitor was determined with casein (Hammarsten, Merck) as substrate according to Kunitz (17). Free elastase activity in puppies serum was determined as described (15) using ^{125}I-elastin as substrate and 1–1.5 ml amounts of serum per incubation mixture. Because of the low levels of activity to be detected, incubation times were prolonged up to 5 hr at 37°.

The following protocol was used for the determinations of the serum inhibitor. Enzyme concentration-activity diagrams were established for elastase and trypsin using 5–100 μg of enzymes. An enzyme concentration was then chosen within the quasi-linear portion of the concentration curves: 25 μg of elastase and 10 μg of trypsin. Using these enzyme concentrations, a serum-inhibition curve was established with increasing amounts of a normal puppy serum (Fig. 1). Both curves obtained have a quasi-linear portion between 10 to 50 μl serum, the midpoint of the curves (50% inhibition) being at 25 μl serum for both

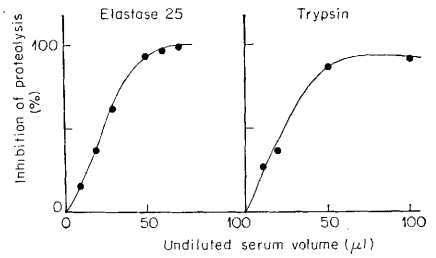

FIG. 1. Inhibition of elastase (25 μg) and trypsin (10 μg) by increasing amounts of a normal puppy serum. Quantity of serum added in μl on the abscissa and inhibition in per cent on the ordinates.

enzymes. The determinations of serum-inhibitors in the treated puppies sera were carried out as follows:

(a) *Elastase inhibitor:* 1 ml of a suspension containing 10 mg of ^{125}I-elastin was pipetted in 25 ml Erlenmeyer flasks, 25 μl of serum was added to this suspension, mixed, then 25 μg of elastase in 25 μl distilled water and the volume was brought to 3 ml with Tris–HCl buffer, 0.02 *M* pH 8.6.

The flasks were incubated for 30 min at 37° with constant shaking. Control flasks with no serum and no elastase were always included. 1 ml samples were withdrawn after 30 min of incubation, filtered on Whatman No. 40 filter paper, rinsed twice with 1 ml H_2O and the radioactivity of the pooled filtrates was determined on an X-ray spectrometer (15).

Activities were corrected for the radioactivity of the elastin sample incubated without enzyme. This background radioactivity is less than 10% of the total radioactivity of the elastin sample "dissolved" with elastase, if care is taken to eliminate daily, free iodine-125 with repeated washing and centrifugation of the labelled elastin suspension. Inhibition was calculated from the difference of counts of the samples with and without inhibitor and expressed as per cent of the uninhibited activity ($i\%$). The decrease of the serum inhibitor titer in the treated puppy serum was expressed as the difference of inhibition before and after elastase injection ($\Delta i\%$).

(b) *Trypsin inhibitor:* 2 ml of 1% casein dissolved in 0.1 *M* phosphate buffer, pH 7.6, were mixed with 25 μl serum, 10 μg of trypsin dissolved in 10 μl H_2O were added followed by 0.1 *M* phosphate buffer pH 7.6 brought to $-$q 4.0 ml final volume. This mixture was then incubated at 37° for 30 min. Another variant of this protocol was used alternatively in order to check the possibility of a trypsin catalysed degradation of the serum inhibitor. This protocol is the following:

2 ml of a 0.1 *M* phosphate buffer pH 7.6 were pipetted into 25 ml Erlenmeyer flasks. 10 μg trypsin dissolved in 10 μl H_2O were added followed by 25 μl serum, mixed, incubated for 10 min at room temperature

and 2 ml of a 1% casein solution in the same phosphate buffer were then added.

After 30 min of incubation at 37° with constant shaking 2 ml samples were withdrawn and precipitated with 2 ml 5% trichloracetic acid. After 60 min at room temperature the tubes were centrifuged and the optical density of the supernatant read at 280 nm. Controls included a casein solution incubated with trypsin in the absence of serum, and casein incubated alone. Both protocols gave identical results suggesting that the degradation of the inhibitor by trypsin during the 30 min incubation was not significant.

Results. When elastase is injected iv the serum inhibitor level to elastase shows a temporary drop followed by a gradual return towards the original value. No such drop of the inhibitor level is found to trypsin. Figure 2 shows a typical time curve for the serum elastase and trypsin inhibitor levels following the iv injection of 20 mg and 100 mg of elastase. A drop of the elastase inhibiting titer of the serum can be noticed which is not accompanied by a concomitant drop of the trypsin inhibitor level. No significant variation of either elastase or trypsin inhibitor levels were found after the iv injection of saline (Fig. 3).

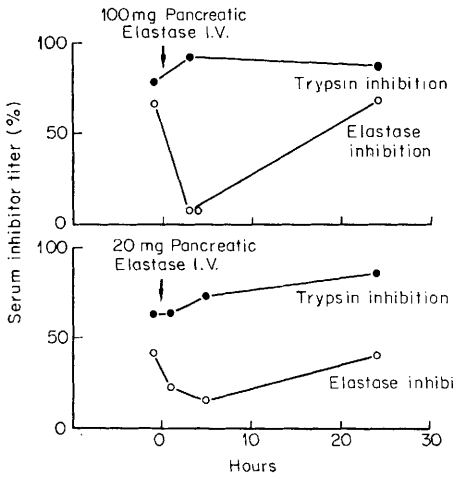

FIG. 2. Drop of serum elastase and trypsin inhibitor titers after intravenous injection of 20 mg and 100 mg of elastase. Abscissa: time in hours. Ordinates: % inhibition of elastase and trypsin. The arrow indicates the time of injection of elastase. ○—○ elastase inhibitor titer, ●—● trypsin inhibitor titer.

The serum elastase inhibitor level drops consistently, but does not disappear completely even after the highest amounts of enzyme injected (200 mg). The drop of the serum elastase inhibitor titer is relatively slow. The maximal drop occurs at about 3–5 hr after elastase injection depending on the amount of enzyme used (Fig. 2). With less than 150 mg of enzyme injected the initial value of inhibitor concentration is restored after 24 hr but only a partial recovery of the normal inhibitor level is observed 24 hr after the injection of high doses of enzyme (150–200 mg).

Table I shows the average values of the elastase and trypsin inhibitor titers in the untreated, normal puppy sera together with the standard error of the mean. A relatively large dispersion of the normal values was observed, the standard deviations (±16) being 35% of the average for the elastase inhibitor titers and 40% for the trypsin inhibitor titer (±25). The reason for these variations of the normal values is unknown. No comparable dispersion of trypsin or elastase titers was observed in normal human sera studied simultaneously (18).

Table I shows also the lowest inhibitor titers to elastase and trypsin observed in puppy sera after the iv injection of increasing amounts of elastase. It can be seen that

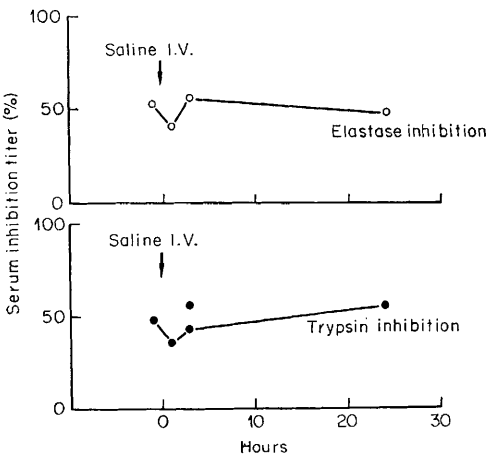

FIG. 3. Same as Fig. 2 but saline was injected instead of elastase. ○—○ elastase inhibitor titer, ●—● trypsin inhibitor titer.

TABLE I. Average Values of Normal Puppy Serum Elastase and Trypsin Inhibition Calculated from Results Obtained with 11 Different Sera ± Standard Error of the Mean and Lowest Values of Inhibition Found After the iv Injection of Increasing Doses of Porcine Pancreatic Elastase.

	$i\%$	
mg elastase injected	elastase	trypsin
0	45.9 ± 4.9[a]	61.9 ± 7.6[a]
20	15.7	63.6
40	12.8	86.4
80	7.0[b]	59.2[b]
100	7.4	88.5
150	4.5	86.8
200	10.8	79.7

[a] Mean values and standard errors of the mean of sera from 4 control animals.

[b] Average of sera from 2 animals injected with this dose of elastase.

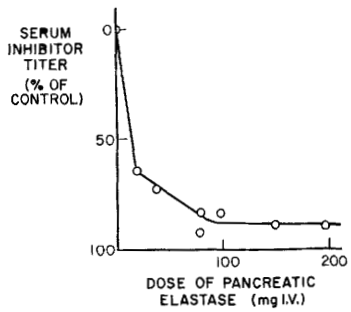

FIG. 4. Maximal drop of serum elastase inhibitor titer ($\Delta i\%$) as a function of the amount of elastase injected. Ordinates: $\Delta i\%$. Abscissa: mg elastase injected.

the elastase inhibitor titers decrease significantly but the trypsin inhibitor titers do not show a similar change.

When the maximal drop of elastase inhibition is expressed as a percentage of the average normal inhibition (45.9%, see Table I) and this parameter, Δi ($\Delta i\% = i_n - i_{el}/i_n$ 100, where i_n is the normal inhibition before injection i_{el} is the lowest inhibition after elastase injection) is represented as a function of the amount of elastase injected, the curve shown on Fig. 4 is obtained. This curve indicates a biphasic reaction between injected elastase and the serum inhibitors. The rapid drop of Δi with the first 20 mg of elastase injected might indicate the combination of the enzyme with inhibitors having a relatively high affinity. This type of inhibitor(s) appears to be saturated at about 20 mg of elastase injected. With higher amounts of enzyme the slope of the saturation curve decreases significantly and flattens out at about 90% Δi, with doses above 100 mg of elastase injected. This second part of the curve can be interpreted as corresponding to the combination of the injected enzyme with inhibitors of low affinity (19).

Elastase activity of puppy sera. No free elastase activity could be demonstrated in any of the serum samples drawn before or after elastase injection, using as much as 1 ml of serum with ^{125}I-elastin as substrate. This would suggest a rapid disappearance of injected elastase from the circulation. Similar results have been obtained for iv injected trypsin and chymotrypsin also, both enzymes disappearing rapidly from the circulation (20).

These results together with the inhibitor data would suggest a disappearance from the circulation of a significant fraction of the enzyme injected with only a part being complexed with serum inhibitors (see Discussion). The fraction eliminated from the circulation may perhaps be adsorbed to elastic fibers of vascular tissues, lung and other organs. Further studies are necessary to elucidate the fate of the injected enzyme.

Histological Effects of Pancreatic Elastase. Modifications of the histological appearance of lung parenchyma and aorta were observed at the lowest dose of intravenous pancreatic elastase administered. These changes consisted of fragmentation and swelling of interalveolar septa with complete rupture of many alveolar walls at the highest doses. Also, in aorta there was a decrease of interfibrillary ground substance in the elastic tissue media. These changes were most marked at the highest doses administered.

Discussion. The intravenous injection of crystalline porcine pancreatic elastase to puppies produces severe alterations of elastic tissue in aorta and lung. This effect may be attributed to the proteolytic action of the injected enzyme, although the possi-

bility of increased release of *in vivo* sources of proteolytic enzymes cannot be excluded (22). Similar tissue lesions were observed in animals in experimental preparations (1, 2). In man possible alterations in elastic tissue have been implicated in pulmonary emphysema secondary to a deficient serum inhibitor level, due to a genetic disorder (3, 11, 23).

The results reported here show that the serum inhibitors do not provide complete protection against iv injected elastase, even when the amount of enzymes injected is below the level of the combining capacity of the serum, as calculated from the *in vitro* concentration-inhibition curves. According to such curves (Fig. 1) the serum inhibitor level of a normal puppy should effectively inhibit at least 100 mg of pancreatic elastase (Fig. 1, i.e., 1 ml of normal serum should combine with about 0.5 mg elastase; the injection of 20 mg elastase could produce a maximal serum concentration of only about 0.10 mg/ml in a 4 kg puppy assuming a 5% plasma volume). 20 mg of the enzyme did however produce noticeable tissue alterations (21).

The higher levels of enzyme injected (above 20 mg) produce less of a decrease of the serum inhibitor level ($\Delta i\%$) than the first 20 mg of elastase injected (Fig. 4). This can be attributed to a relatively loose combination with serum inhibitor(s) having a lower affinity for the enzyme. This finding is in agreement with the more severe tissue alterations observed with the higher doses of enzyme injected (21).

The observed decrease of the serum-elastase inhibitor level was slow and incomplete even with the highest doses of enzyme (150–200 mg) injected. This may be attributed to factors such as the rapid adsorption of the injected enzyme on the elastic fibers of blood vessels and other tissues as previously demonstrated (24, 25), to the occurrence of "temporary inhibition" (26), to proteolytic attack of the inhibitor by the combined elastase or to an enzyme-inhibitor complex(es) of low affinity.

The data on the maximal drop of serum inhibitor titer $\Delta i\%$ as a function of elastase injected (Fig. 4), suggest the presence of inhibitors with a high affinity for elastase and inhibitors with a lower affinity for the enzyme. The former ones would be saturated at about 20 mg of elastase injected, whereas the low affinity inhibitors appear to be saturated to about 100 mg enzyme injected. Therefore at higher than 20 mg elastase injected the enzyme present above this level might be more loosely combined than the first 20 mg.

These data indicate that the fraction of total injected enzyme uncombined may be significant at medium doses of elastase injected (above 20 mg) and very high at the high doses (above 100 mg). According to these results the low affinity of the puppy serum inhibitors to porcine pancreatic elastase can explain the observed pathological effects (21). It should be stated however that the results shown here may not apply to other species since species differences in serum inhibitors have been observed (27). Pancreatic elastase may differ also from tissue elastases more directly involved in the pathogenesis of elastic tissue breakdown.

A further conclusion which can be drawn from the present experiments concerns the relationship between serum inhibitors to elastase and trypsin. No parallel variation could be observed in these two serum-inhibitory-activities, in contradistinction to the good parallelism observed in *in vitro* experiments (9). The trypsin inhibitor titer did not follow the elastase inhibitor titer at any time, even with high amounts of elastase injected. This result disagrees with former findings suggesting a close relationship between trypsin and elastase inhibitors in serum (3, 9, 10). Two of the 5 purified human serum trypsin inhibitors investigated by Heimburger and Haupt (10) were active against pancreatic elastase also (α_1-antitrypsin and α_2-macroglobulin). No purified serum fraction was reported active against elastase and inactive against trypsin. Our results would indicate however that in *in vivo* conditions at least such selective inhibition may occur.

Some conclusions may be drawn from the present data as to the rate of restoration of elastase inhibitors. Their normal level was

reestablished in about 24 hr after the injection of low to middle doses of elastase (less than 150 mg). However, only 50% recovery was observed with high doses. These experiments do not allow distinction between the resynthesis of inhibitors in this interval or their transfer into the circulating blood from extra-vascular sources.

Summary. Varying amounts of crystalline porcine pancreatic elastase were injected iv into puppies in order to study its effect *in vivo* on elastic tissue (21) and on serum elastase inhibitors. Serum inhibitors to pancreatic elastase and to trypsin, were determined in blood samples drawn at intervals after injection. The serum elastase inhibition level decreased 3–5 hr after elastase injection. The decrease was a function of the amount of enzyme injected and reached the highest levels at 80–200 mg elastase injected. The decrease of elastase inhibitor titer with injected elastase suggests a dissociable enzyme-inhibitor complex(es). No parallelism was observed between serum inhibitor titers to elastase and to trypsin. The trypsin inhibitor titer dropped only exceptionally and did not follow at any time the kinetics of the elastase inhibitors. The elastase inhibitor titer returned to normal 24 hr after injection of 20–150 mg of enzyme but only partial recovery was observed in this interval with higher doses. The relatively low affinity of the serum inhibitors to elastase may explain the tissue lesions observed even at low doses of elastase injected (20 mg).

The authors express their deep appreciation to Dr. Jean Cachera and Dr. Jacques Hinglais for making available for these animal studies the excellent facilities of the Laboratoire D'Etude de Technique Churgical, CNRS of Hôpital Broussais, Paris. Appreciation is also expressed to M. Jean P. Gascard and Mlle. Jackeline Hoerter for their fine technical assistance.

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Received Mar. 5, 1974. P.S.E.B.M., 1974, Vol. 146.