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Characterization of Elastase Associated With Granulomatous Tissue Remodeling

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Elastases have been reported to be involved in various types of tissue injury. In this study we detected hydrolytic activities for [^{3}H]-elastin and Suc-Ala-Ala-Ala-pNA (SLAPN) in hepatic granulomas which became elevated in parallel with enlargement of the granulomas and disappearance of aldehyde-fuchsin-stained filaments in the lesions of mice infected with *Schistosoma mansoni*. The elastase was partially purified by gel filtration followed by anion-exchange chromatography. This enzyme has a molecular weight of 20–25k and hydrolyzed denatured collagen (azocoll), Glu-Pro-Val-pNA, SLAPN, and [^{3}H]-elastin. Optimal pH was 7–8.5. It is a serine proteinase and distinct in its inhibitor profile from murine peritoneal macrophage elastase, which has been reported by others. Digestion of elastic fibers in vessel walls and fine fibrils in newly developed granulomas by the granuloma elastase was histochemically identified with aldehyde-fuchsin stain. These results indicate that a serine proteinease functions as a major elastase in granulomatous tisssue remodeling and may account for the disappearance of elastic fibers and other elements of the matrix in fully developed granulomas.

Key words: granulomatous inflammation, murine elastase, aldehyde-fuchsin-stained fibers, granuloma

Elastases from neutrophils, monocytes, and macrophages have been considered to be involved in tissue remodeling associated with connective tissue damage [1,2]. Elastase in human neutrophil granules purified by Ohlsson and Olsson [3] is released in response to activated complement factors or bacteria [1] and has been shown to digest not only elastin but also proteoglycans [4], collagen [5], and fibronectin [6]. The enzyme appears to be partially responsible for tissue injury in pulmonary emphysema [7] and other conditions such as vasculitis, arthritis, and glomerulonephritis [1]. Human monocyte-elastase-like enzyme has plasminogen-independent fibrinolytic activity [8] and degrades serum amyloid protein A [9,10]. Senior et al [11] purified

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monocyte elastase from U-937 cells and found that it is a serine protease similar to neutrophil elastase.

Secretion of elastase by murine alveolar macrophages [12] and thioglycolate-stimulated peritoneal macrophages [13] has been reported. The peritoneal macrophage elastase is a metalloproteinase [14] and catalytically distinct from other elastases [2] as it does not cleave valine or alanine ester substrates [14], frequently used substrates for granulocytes [15], and pancreatic elastase [16]. Focal accumulation of macrophages, monocytes, and granulocytes which causes local tissue destruction and remodeling occurs in granulomatous disease [17]. Secretion of elastase by macrophages isolated from carageenan-induced granulomas in mice was commented on by Bonney [18]. Other laboratories [19] and our own [20,21], using murine granuloma models, also reported that elastase may be involved in the tissue remodeling.

This paper describes properties of partially purified elastase from granulomas experimentally developed in mice. In addition, the native substrates for the enzyme were histochemically examined in the tissues in which macrophages organize to form granulomas.

MATERIALS AND METHODS

Chemicals

N-succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide (SLAPN) was purchased from Peninsula Lab (Belmont, CA): L-pyroglutamyl-L-propyl-L-valine-p-nitroanilide (S2484) was from Kabi Diagnostica (Stockholm, Sweden); azocoll was from Calbiochem (La Jolla, CA); Sephacryl S-300, DEAE-Sepharose, and the calibration kit for molecular weight determination were from Pharmacia Fine Chemicals (Piscataway, NJ): myoglobin was from Serva Feinbiochemica (Heidelberg, West Germany): elastin from bovine nuchal ligamentum, inhibitors, porcine pancreatic elastase (type III), and collagenase (type II) were from Sigma (St. Louis, MO).

Animals and Isolation of Granulomatous Lesions

About 50 cercariae of the Puerto Rican strain of *Schistosoma mansoni* were subcutaneously injected into male BALB/c mice (20–25 g body weight), Harlan Sprague-Dawley Inc. (Madison, WI). After 8, 10, 12, and 13 wk, livers were removed from infected mice and the granuloma-rich and granuloma-poor fractions were separated as previously described [22]. Livers were also removed 6 wk after injection; granulomas had not developed by this period and the whole liver was used. Livers of infected mice were also fixed in 4% formalin and paraffin sections were prepared for histological observation.

Enzyme Assay

Elastinolytic activity was measured by using [^{3}H]-elastin according to the method of Banda and Werb [14]. The specific activity was 1.07×10^5 cpm/mg elastin. Each reaction mixture, final volume 0.3 ml, contained 0.1 ml of tissue sample, 600 μg of [^{3}H]-elastin, 1 mM $CaCl_2$, and 0.1 M glycine-NaOH buffer, pH 8.5, in a disposable, 0.4 ml microcentrifuge tube. It was incubated for 24 hr at 37°C, centrifuged in a Beckman microfuge, and radioactivity solubilized in the supernate was counted. One unit of elastase activity was defined as the solubilization of 1 μg of [^{3}H]-elastin/hr at 37°C. Elastase assay with synthetic substrates SLAPN and S2484

were performed according to modified methods of Bieth et al [23] and Izaki et al [15,24], respectively. The reaction was started by adding 0.05 ml of either 10 mM SLAPN or 4 mM S2484 to 0.1 ml of preheated sample diluted with 0.75 ml of 0.1 M Tris-HCl buffer, pH 8.0. They were incubated for 5–24 hr at 37°C. After the addition of 0.1 ml of 50% acetic acid, absorbance of free p-nitroaniline was measured at 405 nm. One unit of enzyme was defined to liberate 1 μmol of p-nitroaniline (10,500/M·cm). Nonspecific proteolytic activity was determined with azocoll as the substrate [25]. Each assay tube contained 0.2 ml of enzyme solution or buffer as a control, and 0.2 ml of saline was added with 2 mg of azocoll suspended in 0.4 ml of 0.2 M Tris-HCl buffer, pH 8.0. Tubes were shaken during incubation for 2–24 hr at 37°C and centrifuged at 1,000g for 10 min to separate the supernate. Absorbance of the solubilized reaction product at 520 nm was measured. For pH optimum study, acetate buffer, pH 5, phosphate buffer, pH 6, 7, and 7.4, and Tris-HCl buffer, pH 8 and 9, were used.

Extraction of Elastase

Whole livers at 6 wk and granuloma-rich fractions at 8, 10, 12, and 13 wk were homogenized with a glass tissue grinder in 10 times volume of 0.05 M Tris-HCL buffer containing 0.1 M NaCl, 100 units/ml penicillin, and 100 μg/ml streptomycin, pH 8.0. Supernate (soluble fraction) was separated after centrifugation at 20,000g for 20 min and precipitate was suspended in 10 times volume of 0.1% (v/v) Triton X-100 H_2O containing 2 M KSCN, homogenized with a Polytron, and frozen in acetone and dry ice. After three cycles of freezing and thawing, the suspension was centrifuged at 100,000g for 30 min and the supernate was dialyzed against 0.05 M Tris-HCl buffer containing 1 M NaCl and 0.1% Triton X-100, pH 8.0. This fraction is called the membrane-bound fraction. Protein concentration was determined by the method of Lowry et al [26], with bovine serum albumin as the standard. Specific activities of the enzymes were calculated as unit/mg protein. Data were expressed as mean ± SEM and differences were considered significant at P values lower than .05 by Student's t test.

Partial Purification of Elastase

Membrane-bound fraction from a granuloma-rich fraction of 10 wk liver was filtered through Millipore membrane of 0.45 μm pore (Millipore, Bedford, MA) and applied to a Sephacryl S-300 column (2.6 × 70 cm) which was equilibrated and eluted at 4°C with 0.05 M Tris-HCl buffer containing 1 M NaCl and 0.1% Triton X-100, pH 8.0. The column was calibrated by a calibration kit and myoglobin. Fractions with elastase activity were concentrated, dialyzed against water, lyophilized, and kept at 4°C until use. The sample was dissolved in 0.02 M Tris-buffer containing 0.1% Triton X-100, pH 8.0, and applied to a DEAE-Sepharose column (1.6 × 20 cm) equilibrated with the same buffer. The protein was eluted out with a linear gradient of 0 to 1 M NaCl.

Inhibitor Profile

Diisopropylfluorophosphate (DFP), antipain, and chymostatin were dissolved in dimethylsulfoxide (DMSO), phenylmethylsulphonyl fluoride (PMSF) in methanol, 1,10-phenanthroline in ethanol, while other inhibitors and normal mouse serum were prepared in saline. Mixtures of enzyme solution and inhibitors were incubated at

room temperature for 30 min before adding the substrate. The final concentration of each inhibitor used to test the effects on purified enzyme is shown in Table I. Residual enzyme activity was expressed as a percentage of activity in the solvent controls.

Histology

Paraffin sections of mouse livers were stained with hematoxylin and eosin, aldehyde-fuchsin [27], Weigert's resorcin-fuchsin [28], and Verhoeff's elastin stain [29]. The effects of enzymes on fibers in granulomas detected with aldehyde-fuchsin were studied in livers at 7 and 10 wk after infection. The tissue sections were deparaffinized and hydrated. They were incubated with the enzyme solutions or buffer alone in a moist chamber for 24 or 48 hr at 37°C. The sections were then rinsed in water and stained with aldehyde-fuchsin. The enzyme used was the elastase fraction from 10-wk granulomas separated by a Sephacryl S-300 column. The enzyme was concentrated to the azocollytic activity of 0.175 or 0.350 ΔA_{520}/hr. Porcine pancreatic elastase, type III, and collagenase, type II, were dissolved in 0.05 M Tris-HCl buffer, pH 8.0, with 1 M NaCl and 0.1% Triton X-100 and diluted to the same azocollytic activities.

RESULTS

Total Elastase Activity

Hydrolytic activity for [^{3}H]-elastin was low in either soluble (0.7 ± 0.02 units/mg protein) or membrane-bound (6.0 ± 0.7 units/mg protein) fractions prepared from the liver of mice 6 wk after infection. Similarly, enzyme activity in granuloma-poor fractions from 8–13-wk-infected mice was consistently low (0.8 ± 0.1 units/mg protein). Elastase activites from granuloma-rich fractions assayed with [^{3}H]-elastin and SLAPN are compared with those from livers 6 wk after infection in Figure 1. Soluble fractions exhibited low elastase activity (3.2 ± 0.1 units/mg protein at 8 wk; 3.0 ± 0.5 units/mg protein at 10 wk). In contrast, proteinase activity of membrane-

TABLE I. Effect of Inhibitors on Elastase Extracted From Granulomas*

	Azocollysis		(^{3}H)-elastinolysis		SLAPN hydrolysis	
Inhibitors	(fc)[a]	Activity (%)	(fc)	Activity (%)	(fc)	Activity (%)
DFP	(1 mM)	0	(3.3 mM)	3	(1 mM)	6
PMSF		ND[b]	(2 mM)	7	(1 mM)	12
SBTI[c]	(1 mg/ml)	4	(0.3 mg/ml)	12		ND
Aprotinin	(1.000 KIE[d]/ml)	130	(333 KIE[d]/ml)	128	(500 KIE[d]/ml)	100
Elastatinal	(50 μg/ml)	84	(33 μg/ml)	125	(10 μg/ml)	105
Antipain	(50 μg/ml)	196	(33 μg/ml)	159		ND
Chymostatin		ND	(33 μg/ml)	127		ND
Iodoacetamide	(1 mM)	93	(3.3 mM)	146		ND
1,10 phenanthroline	(1 mM)	84	(3.3 mM)	125	(1 mM)	71
Mouse serum	(0.5%)	12	(3.3%)	15.5		ND

*SLAPN, Snc-Ala-Ala-Ala-pNA; DFP, diisopropylfluorophosphate; PMSF, phenylmethylsulphonyl fluoride.
[a]fc, final concentration.
[b]ND, not determined.
[c]SBTI, soybean trypsin inhibitor.
[d]KIE, kallikrein unit.

bound fractions increased (15.9 ± 3.7 units/mg protein) significantly by 8 wk after infection. The increase appeared to plateau between 10 wk (31.5 ± 3.7 units/mg protein) and 13 wk (31.6 ± 11.5 units/mg protein) after infection. Values in parenthesis are [^{3}H]-elastin digestion, but the enzyme activity measured with SLAPN showed a similar pattern.

Partial Purification

A typical elution pattern of the membrane-bound fraction from a Sephacryl S-300 column is shown in Figure 2. Hydrolytic activity for azocoll and S2484 appeared in two peaks: one is about the void volume and the other is at molecular weight 20–25k. Both peaks coincided with fractions with hydrolytic activity for [^{3}H]-elastin and SLAPN (data not shown). The second peak with a higher specific activity (103,594 cpm/mg protein/24 hr) was not detected when the elution buffer did not contain NaCl or detergent. Activity in the second peak was reduced considerably (37% for azocoll, 36% for [^{3}H]-elastin, and 68% for SLAPN) after the fractions were pooled, dialyzed, lyophilized, and redissolved in 0.02 M Tris-buffer, pH 8.0, containing 0.1% Triton X-100 at a concentration of 0.07 mg protein/ml. When this solution was kept at 4°C for 24 hr, about 50% of the activity for [^{3}H]-elastin (49,000 cpm/mg protein/24 hr) and SLAPN was further lost.

Figure 3 illustrates anion-exchange chromatography of the resolubilized sample on DEAE-Sepharose. A single peak with activities for azocoll, [^{3}H]-elastin, SLAPN, and S2484 was eluted at a concentration of about 0.5 M NaCl. The peak fraction was used for determination of pH optimum and inhibitor profiles.

pH Profile

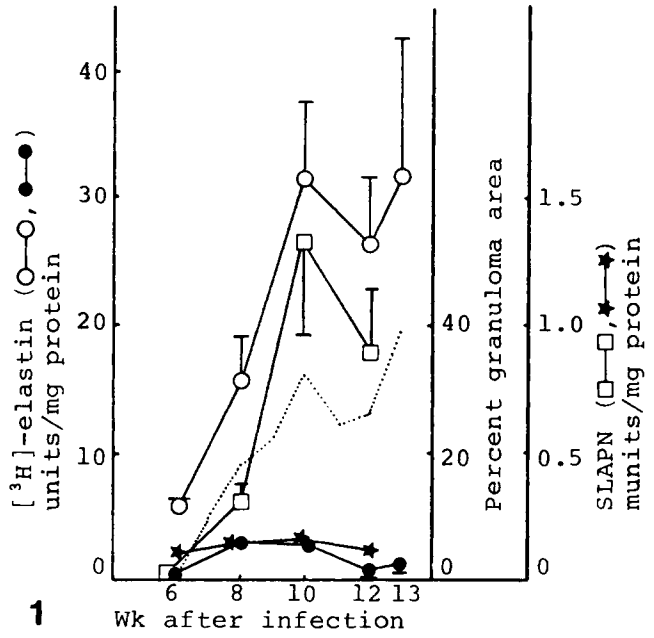
The pH optima were 7–7.4 and 7.4–8.5 for hydrolysis of SLAPN and azocoll, respectively (Fig. 4). The activity was completely abolished at pH below 5 and above 9.

Inhibitor profile

Table I summarizes the inhibition profile for purified elastase from granulomas. The enzyme was strongly inhibited by serine proteinase inhibitors (DFP and PMSF) and mouse serum, but it was not inhibited by cysteine proteinase inhibitor (iodoacetamide). Some inhibitory effect was seen with metalloproteinase inhibitor (1,10 phenanthroline) in azocollysis and SLAPN hydrolysis but not with [^{3}H]-elastinolysis. It was not inhibited by an inhibitor for chymotrypsinlike proteinase (chymostatin), an inhibitor for pancreatic and granulocyte elastases (elastatinal), or inhibitors for trypsinlike proteinase (antipain and aprotinin). Activity in the crude membrane-bound fraction was similarly sensitive to those inhibitors. With [^{3}H]-elastin it was strongly inhibited with serine proteinase inhibitor and the residual activity was reduced to 3% and 5% by 3.3 mM DFP and 2 mM PMSF, respectively. No inhibition was seen with aprotinin, elastatinal, and 3.3. mM 1,10-phenanthroline. However, 22% of the activity was inhibited by chymostatin, indicating that the crude extract contains chymotrypsinlike activity which is not detectable in the partially purified enzyme.

Histological Observations

Hematoxylin and eosin stain of the tissue sections revealed that the granulomatous reaction occurred in livers following essentially the same time course described



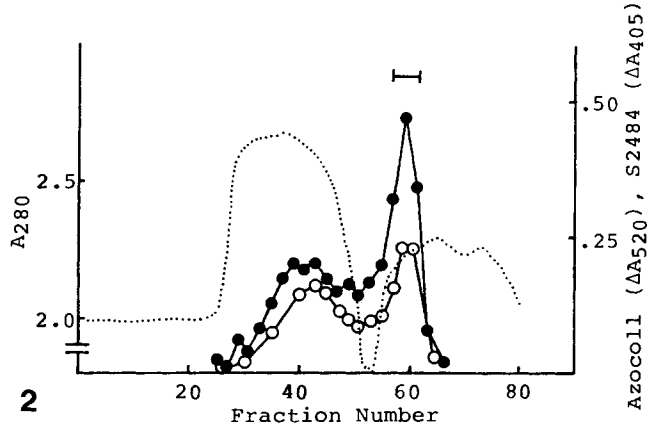


Fig. 1. Elastase activity in extracts from granuloma-rich fractions prepared from livers of mice at different weeks after infection with *S. mansoni*. The values at 6 wk were measured in extracts from whole liver. Activities for [^{3}H]-elastin and SLAPN in soluble fractions (●, ★) and membrane-bound fractions (○, □) were compared with percent granuloma area (. . .) measured morphometrically by Epstein et al [30]. Data are shown as mean and SEM of two (6 and 12 wk), five (8 and 13 wk), or eight (10 wk) mice.

Fig. 2. Gel chromatography of the membrane-bound fraction from hepatic granulomas obtained 10 wk after infection. Assay time was 14 hr for azocoll (●) and 24 hr for S2484 (○). Bar indicates fractions pooled for further purification and histological studies.

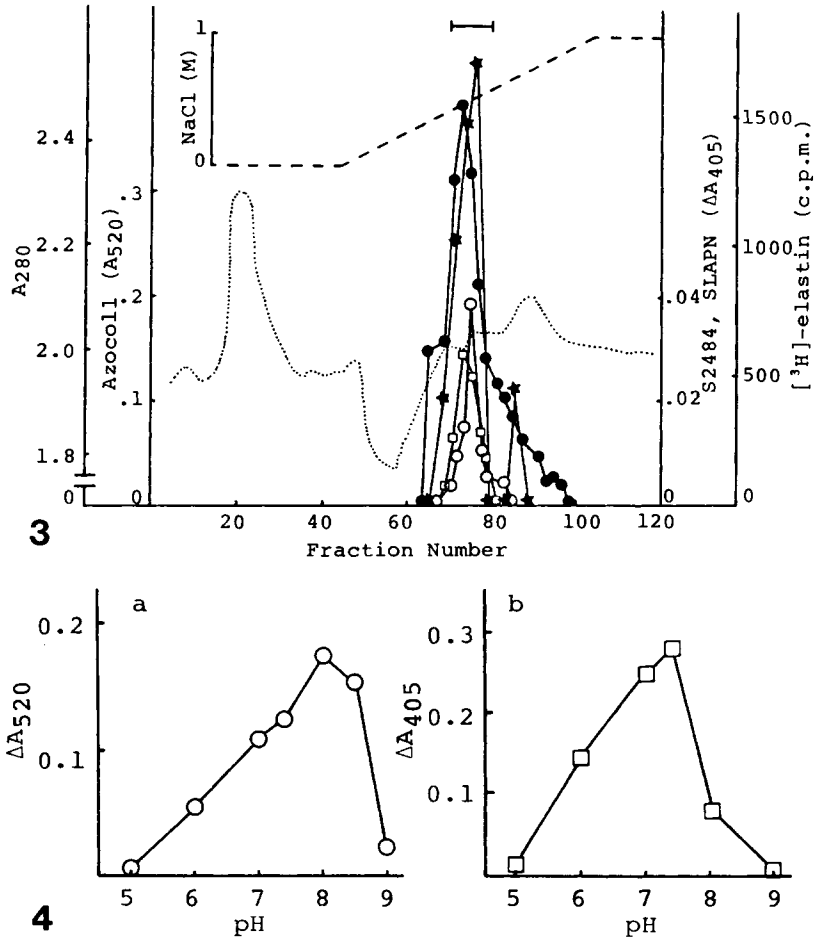


Fig. 3. Anion-exchange chromatography of the elastase in 20–25 k fractions indicated in Figure 2 under the same conditions as described in Materials and Methods. Assay time was 24 hr. Activities for [^{3}H]-elastin (★), azocol (●), S2484 (○) and SLAPN (□) are shown in with A_{280} (. . .) concentration of NaCl (---). Bar indicates fractions pooled for inhibitor profile.

Fig. 4. Effect of pH on the elastase activity, measured with azocollysis (a) and hydrolysis of SLAPN (B). Each value represents the mean of two assays.

by Epstein et al [30]. By 6 wk after infection, small clusters of mononuclear cells and eosinophils were seen in the portal tracts. The three elastic stains used reacted with the internal elastic lamina of arterioles and elastic fibers in the walls of veins. By 7 wk, parasite eggs began to appear in and near small veins. Mononuclear cells and eosinophils accumulated and formed a granulomatous reaction and around the eggs (Fig. 5A). The granulomatous reaction increased by 8 wk and became well organized. By 10–13 wk after infection remnants of the venule ring of elastic fibers were seen in some granulomas and seemed fragmented in some areas. However, the aldehyde-fuchsin stain, but not the other two methods, decorated additional fine filamentous tissue components in 7–8-wk-old granulomas (Fig. 5B). The stained fibrils formed a network configuration among groups of cells in granulomas and extended into the

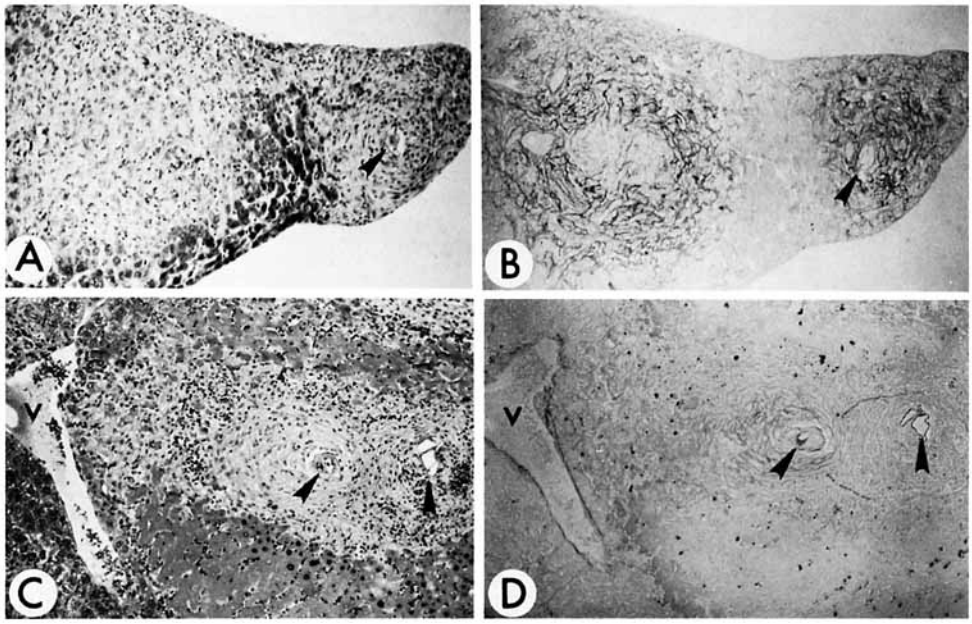


Fig. 5. Granulomatous tissue reaction in murine livers at 7 wk (A,B) and 12 wk (C,D) after infection. Tissue sections were stained with hematoxylin-eosin (A,C) or aldehyde-fuchsin (B,D). Arrowheads eggs; v, vein. Elastic fibers in the wall of the vein are apparently stained in panel D. ×250.

edge of the tissue reaction but did not totally encapsulate the granulomatous response. Aldehyde-fuchsin also stained mast cell granules. As granulomas increased by 9–12 wk after infection (Fig. 5C), there was a reduction in the number of granulomas which contained the stained filaments (Fig. 5D). Even within one granulomatous lesion, the stained filaments appeared usually only at the periphery and central clearance of staining was noted. Findings from the enzyme digestion study are summarized in Table II. It appears that extracted granuloma elastase, pancreatic elastase, aand commercial and impure collagenase all have the ability to reduce aldehyde-fuchsin staining in 7-wk-old granulomas, but granuloma elastase is most effective in removing the staining of the network of fine fibrils. (Fig. 6).

DISCUSSION

Granulomatous tissue reactions experimentally produced in murine livers showed an elevation of a serine proteinase which is capable of digesting both native and synthetic substrates for elastase. The enzyme concentrates in granulomas and appears to be bound to the membrane by hydrophobic interaction [31] because it is not soluble in Tris-saline but is solubilized in a KSCN solution containing Trition X-100. From the pH optimum and inhibitor sensitivity, properties of the enzyme resembled elastase of human granulocytes [32,33] and monocytes [9,10,11]. The purified enzyme is not a metalloproteinase and differs from previously described mouse elastase [14]. It also does not contain chymotrypsinlike proteinase activity. The molecular weight estimated from gel filtration in the presence of high ionic strength or detergent falls into the range of human as well as mouse elastase. Under a condition of low ionic strength,

TABLE II. Effect of Enzyme Solutions on Intensity of Aldehyde-Fuchsin Staining of Tissue Components in 7- and 10-Week-Old Granulomatous Livers

Solutions tested	Azocollytic activity ($\Delta\Delta_{520}$)	Incubation time (hr)	Elastic fibers in large vessel walls	Granulomatous tissue		
				Vessel fibers	Fine fibrils	Mast cells
Buffer alone	0	24	++	+	++	++
	0	48	++	+	++	++
Elastase	350	24	++	+	+	++
extracted from	350	48	+	−	−	+
10-wk granulomas	175	48	+ ++	−	−	+
Porcine	350	24	++	+	+ ++	++
pancreatic	350	48	+	−	+	++
elastase	175	48	+ ++	±	+ ++	++
Collagenase[a]	350	24	+ ++	±	+ ++	++
	350	48	+	−	+	++
	175	48	+ ++	±	+ ++	++

[a]Collagenase (type II) also contains clostripain, caseinase (nonspecific protease) and tryptic activities.

the enzyme appeared as a high molecular weight mass, most probably a result of binding with other molecules as found by Starkey and Barrett [34] to occur with human spleen elastase. The enzyme was found to be rather unstable after partial purification and further purification was not yet possible.

Specific enzyme activity in granulomas increased with enlargement of granulomatous tissues in the liver indicating that the activity increased with maturation of the tissue reaction. Although the cell source of this enzyme in granulomas remains unknown, macrophages are one potential cell source. Murine macrophages produce metalloproteinase with certain types of stimulation [13] while they may synthesize another type of elastase which is associated with granulomatous tissue remodelling. Alternatively, the elastase was synthesized by other cell types such as neutrophils, and the enzyme, in its active form, was internalized [35], retained [36,37] and released [38] by macrophages as described for human alveolar macrophages. Recently elastase was also detected in human mast cells [39]. However, it is unlikely that cercariae which produce an elastase [40,41] are responsible because of the long delay before the enzyme appears in the liver. In addition, the enzyme is almost certainly not secreted by eggs which contain thiol proteinase [42].

After 9 wk infection elastase activity increased in granulomas at a time when the fine fibril component which stained with aldehyde-fuchsin had already decreased. The findings were interpreted to demonstrate that native substrates for the enzyme localize in granulomas and that the enzyme action may be directly involved in maturation, enlargement, and remodeling of the granulomatous tissue reaction. Histochemical studies comparing the biological action of the granuloma-associated elastase with porcine pancreatic elastase and collagenase showed that the stain of elastic fibers of vessell walls is affected by all three enzyme preparations but the granuloma-extracted enzyme is most effective in removing the fine fibril staining. Thus, regulation in the production of the fine fibrils and a granuloma-associated enzyme which preferentially digests the fibrils appears to be one mechanism that occurs during the remodeling of the matrix during the course of chronic granulomatous inflammation.

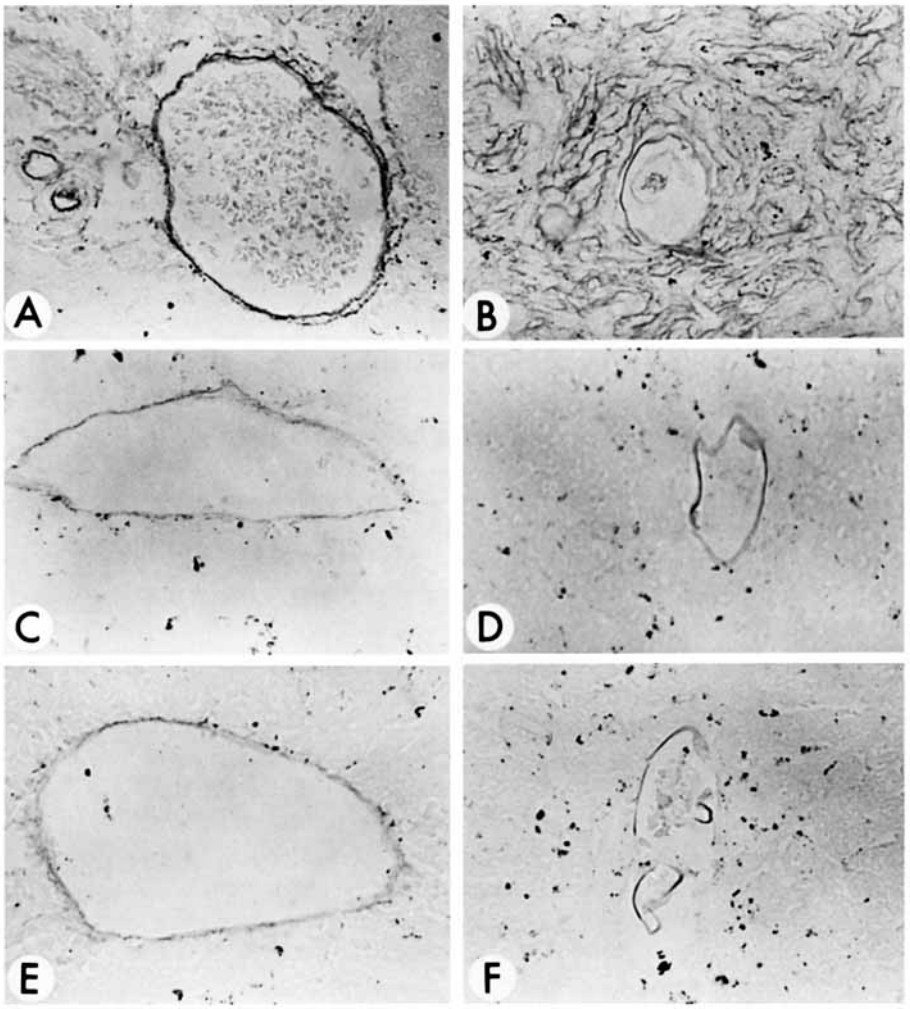


Fig. 6. Effect of enzymes on vessel elastic fibers (A,C,E) and fibrils in granulomas (B,D,F) in livers 7 wk after infection detected by aldehyde-fuchsin. Tissue sections were incubated for 48 hr with buffer alone (A,B), with elastase from granuloma extract (C,D), or with porcine pancreatic elastase (E,F). ×625.

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