MEDIATORS OF INFLAMMATION IN LEUKOCYTE LYSOSOMES

IX. Elastinolytic Activity in Granules of Human Polymorphonuclear Leukocytes*

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Past studies have focused attention on the lysosomal proteinases of polymorphonuclear leukocytes (PMN) as mediators of vascular injury (1–6). A specific association between the hydrolases in these cells and selected targets in vessel walls was suggested by Cochrane and Aiken, who showed that acid-cathepsins D and E extracted from rabbit PMN granules could digest vascular basement membrane in vitro (5). As pointed out by these same authors, acid-cathepsin–mediated vessel damage presupposes significant lowering of local pH. It remains to be determined whether sufficient acidity can be maintained at the tissue-blood interface to permit such reactions to occur in vivo.

Recently, evidence has appeared of vascular damage by leukocyte proteinases with neutral pH optima. A neutral mucoproteinase has been detected in rabbit PMN granules (7), and neutral proteinases have been purified from Arthus edema fluid in this species (8). Human neutrophiles are also known to contain neutral proteolytic activity or "leukoprotease" (9–12). We recently reported that extracts of human PMN granules could degrade vascular basement membrane at physiological pH in vitro and in vivo (6). Indeed, a neutral collagenase has been found in human PMN granules (13) and may be responsible for the lysis of vascular basement membranes occurring at physiological pH.

The present studies were undertaken to examine the neutral proteinases of human neutrophilic leukocytes for possible activity against elastin. This sialoprotein comprises the elastic lamina and supportive fibers present in the walls of arterial and major venous blood vessels. Such activity, if present, would help account for the development of arterial lesions in PMN-mediated arteritis.

Our results show that the granules of human PMN contain neutral elastinolytic activity which can be separated from the collagenolytic activity present in these cells. The data also demonstrate that the properties of elastolysis by

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the PMN agent are sufficiently different from those of elastolysis by pancreatic and serum-elastase as to suggest that the leukocyte factor is a distinct and separate elastolytic entity. In addition, treatment of human tissues in vitro with extracts of human PMN granules will be shown to cause alterations in the elastica-staining properties of arterial vessels resembling the changes associated with acute arteritis.

Materials and Methods

Preparation of Leukocyte Fractions.—Freshly drawn venous blood was obtained from normal male subjects between 20 and 35 yr of age, and was anticoagulated with citrate (75 ml of ACD solution-formula A per 500 ml). The blood was mixed with 2 volumes of cold, 3% dextran (clinical grade H) in isotonic NaCl, and erythrocytes were allowed to settle for 30 min at 4°C. Leukocytes were obtained by centrifugation of the dextran supernatants, in siliconized bottles, at 160 *g* for 20 min. Remaining erythrocytes were then lysed by hypotonic shock in 0.21% NaCl for 30 sec. Lysis was terminated by addition of KCl to a final concentration of 0.15 M. All procedures were carried out between 0° and 4°C. Average yields per 500 ml whole blood equaled 20×10^8 total leukocytes, of which 65–75% were PMN.

After one wash in 0.34 M sucrose, the leukocytes were suspended in a small volume of the same solution (1 ml per 2×10^8 PMN) and mechanically disrupted by vacuum suction through a fine wire screen (0.25 mm^2 openings). A small aliquot of undiluted homogenate was centrifuged at 17,000 *g* × 20 min to obtain a sample of the granule-free, cell sap fraction. The remainder of the homogenate was diluted with excess sucrose solution and was subjected to differential centrifugation. After discarding unbroken cells at 180 *g*, nuclei and granules were collected at 500 *g* and 17,000 *g* respectively. They were then separately resuspended in 0.15 M sodium phosphate buffer (pH 8.0) and disrupted by repeated freeze-thawing. After high-speed centrifugation to sediment debris, the clarified supernatants of lysed nuclei and granules were dialyzed 5 hr against cold, normal saline containing 0.01 M phosphate (pH 7.4). The dialyzed extracts were stored at −60°C until used. The cell sap fraction of the homogenized leukocytes was similarly freeze-thawed, dialyzed, and stored at −60°C.

Blood, "free" of PMN, was also required as a control preparation. In these experiments, freshly-drawn, heparinized human blood was filtered at 37°C through a sterile, nylon-wool column (Leuko-Pak, Fenwal Laboratories, Inc. Morton Grove, Ill.). Differential staining revealed 2% or less of PMN. Platelets, erythrocytes, and lymphocytes were unaffected by this procedure. Thereafter, treatment of the blood was as described earlier.

Pancreatic Elastase (Pancreatopeptidase E, EC 3.4.4.7).—The elastase used in our experiments was a 2X crystallized preparation made from swine pancreas (Worthington Biochemical Corporation, Freehold, N. J.).

Anti-protease Agents.—Soybean trypsin inhibitor was obtained from Worthington Biochemical Corporation. Salivary kallikrein inhibitor (Trasylol, preparation A-128) was obtained from FBA Medical Research Division of Metachem, Inc., N. Y.

Protein Assay.—Protein content of leukocyte fractions and column eluates was measured with the Folin-Ciocalteu reagent according to the method of Lowry (14). Crystalline ("antigen") bovine serum albumin (Pentex Incorporated, Kankakee, Ill.) served as reference standard.

Elastase Assay.—Elastolysis by leukocyte fractions and by pancreatic elastase was measured by the method of Sachar (15), in which dyed, solid elastin is solubilized by the enzyme and released dye is measured colorimetrically. For this purpose, orcein-dyed elastin (Worthington Biochemical Corporation) was employed as substrate at a concentration of 10 mg/ml in the desired buffer. The latter was usually 0.2 M Tris-HCl, pH 8.8 (at 37°C). Other buffer

systems will be described elsewhere in the text. Length of incubation was routinely 2 hr (37°C) unless otherwise specified. Buffer pH was adjusted at the incubation temperature.

In experiments involving measurement of elastolysis in the presence of serum, a modification of the assay method was required to eliminate colorimetric interference by serum pigments. For this purpose, TCA precipitation of serum proteins, followed by extraction of free orcein into butanol, was carried out according to the method of Chao (16). The optical density of the butanol phase was then taken as a measure of elastolysis.

Proteolysis Assay.—Assays of proteolytic activity using denatured hemoglobin as substrate (Nutritional Biochemical Corporation, Cleveland, O.), were carried out according to the method of Anson (17) as modified by Press (18). A 1.7% solution of hemoglobin in 0.06 M sodium phosphate buffer (pH 7 at 37°C) was divided into 1.0 ml aliquots in duplicate assay tubes. Appropriate quantities (See Results) of the materials to be tested were added, and the tubes were incubated for 60 min at 37°C. Reactions were stopped by addition of 0.9 ml of 0.3 M TCA per tube, and the optical density of the TCA supernatants was read at 280 mμ. Absorption blanks were prepared by addition of test materials to substrate tubes after incubation and TCA precipitation steps.

Elastolysis Inhibition Experiments.—

pH: Inhibitory effects of decreasing pH were studied in tris-acid maleate buffer (Trizma maleate, Sigma Chemical Company, St. Louis, Mo.) and in sodium, potassium phosphate buffer. Molarity of both buffers was varied in order to keep ionic strength constant at 0.05 at all pH values. Concentration of test materials are given in the Results section.

Ionic strength: Inhibition by increasing ionic strength was tested in 0.2 M Tris-HCl buffer (pH 8.8 at 37°C) containing different concentrations of NaCl.

Serum: Inhibition by added serum was determined in 0.2 M Tris-HCl buffer (pH 8.8), or in phosphate-buffered saline (pH 7.4). Total ionic strength of the latter system was uniformly kept at 0.1 by mixing a constant volume of phosphate buffer (I = 0.05) with variable quantities of serum and normal saline such that the sum of the volumes of these latter ingredients was also constant and equal to that of the phosphate buffer. Serum concentration varied between 0.1 and 50%. The human serum was either freshly drawn or else had been stored at −20°C for less than 1 wk before use. Orcein liberated by elastolysis in the presence of serum was measured by the butanol extraction method of Chao (16).

Tissue Experiments.—Portions of human kidneys and renal arteries were obtained postmortem. 6-μ thick sections of kidney were cut on a freezing microtome and transferred to glass slides. Control sections were covered with 1–2 drops of normal saline plus 0.01 M phosphate buffer (pH 7.4 at 37°C). Other sections were covered with buffered saline containing 400 μg/ml of PMN granule protein. The slides were placed in moist chambers and incubated at 37°C. At 2-hr intervals, the sections were drained and covered with fresh test solutions. Incubation was continued for a total of 6 hr. Renal arteries were treated as follows: After dissecting away adherent fat and washing in ice-cold saline to remove traces of blood, the vessels were cut into 2-mm wide strips. Individual strips were then incubated in 0.075 M, pH 7.8 tricine buffer (*N*-tris, hydroxymethyl methyl glycine, Calbiochem Corporation, Los Angeles, Calif.). PMN granule protein (450 μg/ml) was added to selected arterial strips, and incubation was continued for 90 min at 37°C.

Following incubation, kidney sections and artery strips were fixed overnight in 10% neutral formalin. The arterial tissue was dehydrated, imbedded in paraffin, and sectioned. Frozen sections of kidney were stained directly. Weigert's resorcin-fuchsin elastic tissue stain was used, and counterstaining was done with van Gieson's solution (19). Parallel kidney sections were also stained with hematoxylin and eosin to facilitate the selection of appropriate vessels for study.

RESULTS

1. Demonstration of Elastinolytic Activity in Human PMN Granules.—Table I summarizes the results obtained when fractions of human leukocyte homogenates and other agents were incubated with orcein-dyed elastin as described in Materials and Methods. Activities of the different materials tested are expressed

TABLE I

Elastolysis by Human Leukocyte Fractions and Other Agents

Test material	Protein concentration	Elastolysis*, ‡
	μg/ml	%
A. Leukocyte homogenate (65–75% PMN)		
Granules	500	100
Nuclei	500	15
Cell sap	500	6
B. PMN-depleted leukocyte homogenate (2% PMN)		
Granules	500	7
C. Purified proteolytic enzymes		
Trypsin	25	0
Chymotrypsin	25	0
Papain	25	0
Elastase§	25	100

* Expressed as per cent of the elastolysis given by 25 μg of pancreatic elastase in the same experiment.

‡ Elastolysis reactions carried out for 2 hr in 0.2 M Tris-HCl buffer (pH 8.8 at 37°C) containing 10 mg orcein-elastin per milliliter.

§ 2 × crystallized pancreatopeptidase E (EC 3.4.4.7) (Worthington Biochemical Corp., Freehold, N. J.).

as per cent of the elastolysis given by a fixed amount of purified pancreatic elastase. Assay conditions were kept constant throughout all experiments. It can be seen from part A of Table I that significant elastinolytic activity was detected in the granule fraction of the leukocyte homogenates. Similar results were obtained with separate granule preparation from 10 leukocyte donors. On an equal weight basis, granule protein consistently possessed about $\frac{1}{20}$th of the activity of the purified pancreatic enzyme. Specific activity of the granules (see Table I) was high in comparison with that of nuclear and cell sap fractions, as was total elastolytic activity of the granule fraction (specific activity X total protein content of the fraction). The latter value was found to be four times greater than total cell sap activity.

Part B of Table I shows that granules prepared from PMN-depleted blood

(see Materials and Methods) were almost entirely devoid of elastinolytic activity, suggesting that the granule fraction of PMN leukocytes is the source of the active agent. In addition to loss of specific granule activity following the elimi-

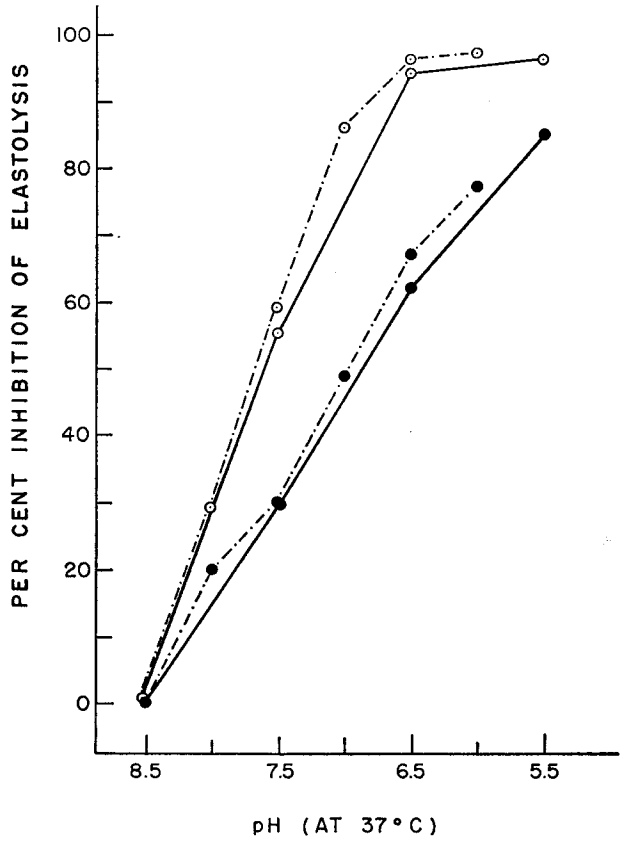

FIG. 1. Effect of pH on elastinolytic activity of pancreatic and leukocyte extracts. Open circles stand for pancreatic elastase (25 μg/ml). Closed circles represent the leukocyte granule extract (450 μg protein/ml). Solid lines indicate assays carried out in tris–acid maleate buffer, and dashed lines represent assays conducted in sodium and potassium phosphate buffer. Ionic strength of both buffers at all pH values = 0.05.

nation of PMN from the starting blood, profound reductions (95%) in total yield of granule protein also occurred.

In part C of Table I it can be seen that the assay system employed was specific for elastinolytic enzymes.

2. Effect of Decreasing pH on the Elastinolytic Activity of PMN Granules and Pancreatic Elastase.—The activity of pancreatic elastase is maximal at pH

8.5–8.8, but becomes inhibited as pH approaches neutrality. This property of the pancreatic enzyme reduces its potential effectiveness as a mediator of elastolysis in acute arterial inflammation. A series of experiments were therefore

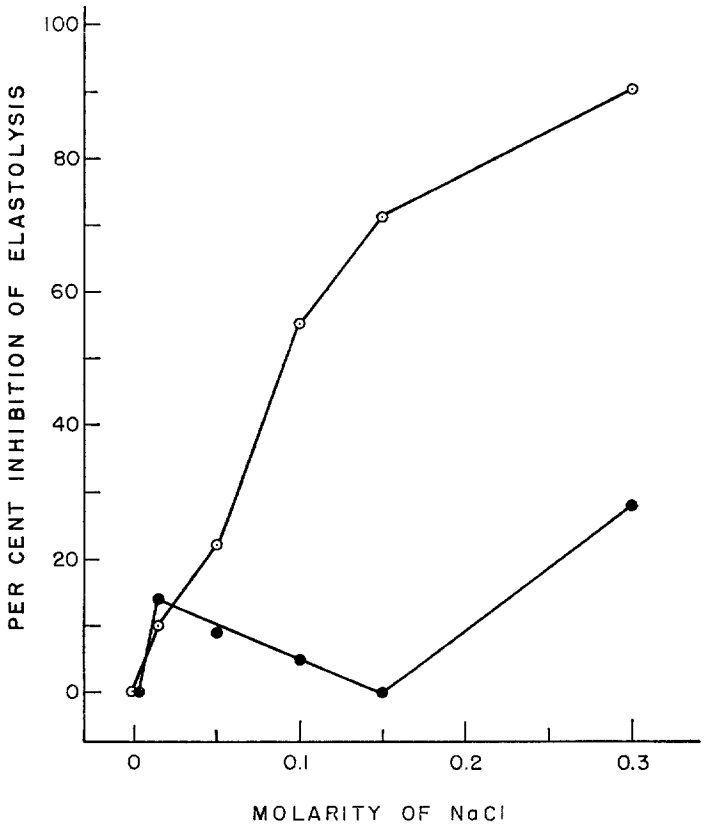

FIG. 2. Effect of ionic strength on elastinolytic activity of pancreatic and leukocyte extracts. Open circles represent pancreatic enzyme (25 μg/ml). Closed circles are the leukocyte granule extract (450 μg protein/ml). All assays were carried out in 0.2 M tris-HCl buffer (pH 8.8 at 37°C) containing variable concentrations of added NaCl. Elastolysis at zero molarity of added NaCl was taken as equal to 100%, in calculating per cent inhibition at higher ionic strengths.

performed to determine the effect of decreases in pH on the elastinolytic activity of human PMN granules. Two buffer systems were employed in these experiments and the ionic strength of both buffers was kept constant at all pH values tested. This was necessary in view of the independent inhibition of elastolysis which results from increasing ionic strength (see Results, part 3). Per cent inhibition at lower pH values was calculated in relation to the elastolysis present at pH 8.5, the latter value being arbitrarily taken as 100% activity.

The results are depicted in Fig. 1, in which inhibition of elastolysis by decreasing pH is shown for both the pancreatic and leukocyte agents. It can be seen that in both buffers the PMN granule extract was inhibited about 50% at pH 7 and approximately 75% at pH 6. On the other hand, the pancreatic

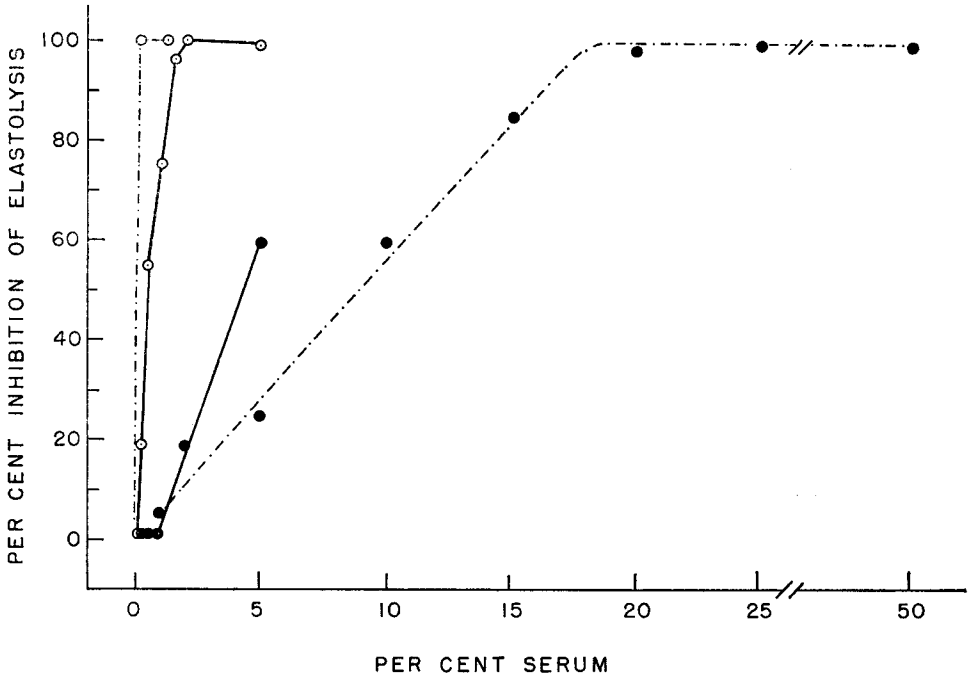

FIG. 3. Effect of human serum on elastinolytic activity of pancreatic and leukocyte extracts. Open circles represent pancreatic enzyme (20 μg/ml). Closed circles represent leukocyte granule extract. Solid line represents incubation in 0.2 M tris-HCl buffer (pH 8.8 at 37°C) plus added serum as shown. Dashed line represents incubation in phosphate saline buffer containing added serum as shown (final ionic strength = 0.1, pH = 7.4). Concentration of leukocyte granule protein is 200 μg/ml in Tris experiment and 400 μg/ml in phosphate saline experiment.

elastase preparation was 80% inhibited at pH 7 and was nearly completely inhibited at pH 6. Thus, at pH values which might be expected to exist in inflamed arterial walls, greater activity is retained by the PMN elastinolytic factor than by the pancreatic enzyme.

3. Effect of Increasing Ionic Strength on the Elastinolytic Activity of PMN Granules and Pancreatic Elastase.—In addition to the inhibitory effect of physiological pH upon pancreatic elastase shown in the preceding section, physiological concentrations of NaCl also inhibit this enzyme (20). Thus in Fig. 2 it can be seen that at its pH optimum (8.8) the pancreatic enzyme is 70% inhibited by an isotonic concentration of NaCl. We therefore investigated the

effect of increasing ionic strength on the elastinolytic activity of leukocyte granules. As seen in Fig. 2, the granule extract was partly inhibited both at low and high concentrations of NaCl, but was completely uninhibited at a physiologic concentration of this salt. This marked difference in the effect of ionic strength on the pancreatic and leukocyte elastinolytic agents lends further support to the suggestion that the latter agent is the potentially more important mediator of vessel wall elastolysis in vivo.

4. Effect of Serum Elastase Inhibitor on Elastolysis by PMN Granules and Pancreatic Elastase.—A potent inhibitor of pancreatic elastase is present in the sera of most animal species, including man (16, 20). We therefore explored the effect of human serum upon the elastinolytic activity of both the pancreatic and the human leukocyte agents. The results are shown in Fig. 3. It can be seen that pancreatic elastase (20 μg/ml) was inhibited 75% by a 1% concentration of fresh human serum in the incubation mixture. Complete inhibition of the action of this enzyme upon elastin was observed in the presence of 2% serum. These reactions were carried out in 0.2 M tris-HCl buffer, pH 8.8 at 37°C. Under the same conditions, leukocyte granule extract (200 μg protein/ml) was not inhibited by 1% serum and was only inhibited 18% in 2% serum. Inhibition of the leukocyte elastinolytic agent was still incomplete (60%) at a 5% serum concentration. These results are even more significant in view of the fact that 200 μg of leukocyte granule protein contains only half the effective elastolytic activity of 20 μg of crystalline pancreatic enzyme (see Results, part 1).

A second serum inhibition experiment (also shown in Fig. 3) was then carried out in a phosphate buffer-saline-serum system (combined ionic strength = 0.1, pH = 7.4, see Materials and Methods). This incubation medium eliminated variations in ionic strength and pH caused by the addition of larger amounts of serum (over 5%) to the tris-HCl buffer used in the previous experiment. Under these conditions, pancreatic elastase was completely inhibited at the start by the combined effects of high ionic strength and neutral pH, without addition of any serum. The leukocytic agent, on the other hand, again demonstrated greater resistance to the action of serum-elastase inhibitor (see Fig. 3).

Thus, the leukocyte granule elastinolytic factor appears to be significantly less susceptible to the action of circulating elastase inhibitor than is the pancreatic enzyme. This observation, as well as the findings presented in the sections on pH inhibition and ionic strength inhibition, provide arguments favoring the leukocyte as a major source of pathological elastolytic activity.

5. Effect of Antiprotease Agents on the Elastinolytic Activity of PMN Granules and Pancreatic Elastase.—Soybean trypsin inhibitor and salivary kallikrein inhibitor are reportedly ineffective against the elastinolytic activity of pancreatic elastase, although these same antiprotease agents can block the nonspecific proteolytic activity associated with the enzyme (20). When the above-

mentioned inhibitors were also tested on human PMN granules, marked inhibition of elastolysis was observed. These results are summarized in Table II. As shown, small quantities of pancreatic enzyme (5 μg/ml) retained full elastolytic activity in the presence of relatively large amounts of inhibitory agents. In contrast, PMN granule activity was inhibited 50% by the highest concentrations of the same inhibitors. All reactions were conducted in tris-HCl buffer under optimal conditions for elastolysis (low ionic strength and alkaline pH).

6. Effect of Ionic Strength, Serum, and Antiprotease Agents on the Hemoglobin-Digesting Activity of Human PMN Granules and Pancreatic Elastase.—We next

TABLE II

*Effect of Antiprotease Agents on Elastolysis**

Test agent	Concentration	% inhibition‡	
		Pancreatic elastase (5 μg/ml)	PMN granule extract (450 μg protein/ml)
Soybean trypsin inhibitor, μg/ml	50	0	19
	100	0	24
	500	0	49
Salivary kallikrein inhibitor, units/ml	200	0	0
	2000	0	49

* Reactions carried out in 0.2 M tris-HCl buffer (pH 8.8 at 37°C) containing 10 mg orcein-dyed elastin per milliliter.

‡ Based on values of orcein released in the absence of protease inhibitors.

tested increasing ionic strength and fresh human serum for their effects on the hemoglobin-digesting activity of PMN granules and pancreatic elastase. The results are shown in Table III. As expected, concentrations of saline which interfered significantly with elastolysis by the pancreatic preparation (see part 3 of Results) had no discernible effect on the hemoglobin-digesting activity of the same preparation. Neither did these same concentrations of salt inhibit hemoglobin digestion by PMN granule extracts. Again, as expected, addition of fresh serum inhibited proteolysis by the pancreatic elastase complex, and the same concentrations of serum also inhibited (although to a lesser extent) the proteolytic activity of PMN granules. Finally, as shown in Table III, soybean trypsin inhibitor also partially inhibited hemoglobin digestion by PMN granule extracts.

7. Preliminary Separation of Elastinolytic and Collagenolytic Activities of Human PMN Granules.—Elastin is not significantly affected by any known protease other than elastase. Nevertheless, we attempted to distinguish the elastinolytic agent of human PMN granules from the collagenase also present

in these particles (13). A preliminary separation of the two activities was achieved by using ion-exchange, column chromatography on CM-Sephadex.

21 mg of granule protein were concentrated into a volume of 1 ml and dialyzed against 0.01 M barbital buffer, (pH 8.46 at 4°C). Precipitated material was removed by centrifugation and the clarified sample was placed on a 0.9 × 15 cm column containing 2.0 g of CM-Sephadex (C-25) previously equilibrated with the buffer. After two complete bed volumes of the buffer had passed through the column, a step-wise gradient of NaCl in barbital buffer was started. All procedures were carried out at 4°C. Protein eluting from the column was monitored with the Folin assay.

TABLE III

Effect of Various Inhibitors on Proteolysis by PMN and Pancreatic Elastinolytic Extracts*

Inhibitor	Concentration	% inhibition‡	
		Pancreatic elastase (4 μg/ml)	PMN granule extract (40 μg protein/ml)
NaCl, M	0.15	0	0
	0.30	0	0
Human serum, %	1	81	60
	5	93	60
Soybean trypsin inhibitor, μg/ml	50	0	0
	500	0	63

* Hemoglobin digestion assay as described in Materials and Methods.

‡ Based on values of TCA-soluble peptides released in the absence of inhibitors.

An initial peak containing 1.7 mg of protein appeared just after the column-void. This peak contained no detectable elastinolytic activity and only a negligible amount of hemoglobin-digesting activity, but accounted for half of the total collagenase present in the starting granule extract (bovine Achilles tendon substrate; ninhydrin assay). Additional protein was then recovered in four broad peaks eluting between 0.1 and 0.45 M NaCl. Elastinolytic and hemoglobin-digesting activities were found in the fractions eluting with 0.45 M NaCl. These observations, although preliminary, suggest that the collagenase and the elastinolytic agent of human PMN granules are separate entities. The further purification of PMN-elastase will be reported elsewhere.[1]

8. Effect of PMN Granule Extracts on the Elastica-Staining Properties of Human Arteries In Vitro.—Frozen sections of human kidney and strips of

[1] Janoff, A. Mediators of inflammation in leukocyte lysosomes. X. PMN elastase: partial purification and further study of effects upon elastic tissue. Manuscript in preparation.

human renal artery were incubated with human PMN granule extracts as described in Materials and Methods. Control tissues were incubated in buffers alone. After designated intervals, the tissues were fixed and stained for elastic fibers (see Materials and Methods). A considerable reduction in the intensity of elastica staining was consistently observed in muscular vessels of intermediate caliber in kidney sections exposed to the action of the granule extract. Representative results are shown in Figs. 4 *b* and 4 *d*. Many smaller caliber arteries were almost completely denuded of their elastic lamellae and could only be observed with difficulty in sections stained for elastic tissue. In general, the same vessels were compared in adjacent tissue sections (see Figs. 4 *a*–4 *d*). Relatively short exposure of renal artery strips to the action of the granule extract frequently resulted in partial fragmentation of the internal elastic lamina and separation of this layer from the underlying smooth muscle cells (see Figs. 5*a*–*d*).

Thus, treatment of human arterial vessels in vitro with extracts of human PMN granules caused alterations in elastic elements resembling those found in PMN-mediated arteritis. A comparison of the effects of purified PMN and pancreatic elastases upon arterial elastic tissue under physiologically inhibitory conditions (serum, ions, pH) will be the subject of a separate report.[1]

DISCUSSION

Pancreatic elastase is not only capable of carrying solid elastin into solution, but is also capable of digesting a variety of soluble protein substrates (e.g. hemoglobin, casein, fibrin). Ling (21) has recently separated the two activities into a specific elastinolytic enzyme and a second enzyme with broad, nonspecific proteolytic activity. The latter, however, demonstrates relatively low affinity for many synthetic substrates of proteolysis. Human PMN granule extracts also digest hemoglobin and fibrin at neutral pH and yet show little activity against several synthetic substrates of proteolysis (6). Moreover, this nonspecific proteinase activity of PMN granule extracts is associated with the elastinolytic and not the collagenolytic fractions of the extract, when these are separated as described earlier. In the foregoing respects, "leukocyte-elastase" resembles the elastase complex present in pancreatic tissue.

Despite the above-mentioned similarity, the data presented in this report show that the elastolysis reactions mediated by human PMN granule extracts and porcine pancreatic elastase can be distinguished in several ways. The two reactions clearly differ with respect to pH effects, salt inhibition, and sensitivity to inhibition by serum or antiprotease drugs. These observations suggest that leukocyte granules contain an elastinolytic factor different from that which is present in pancreas (assuming that human pancreatic elastase has properties similar to those of the pancreatoelastases of other species). Serum elastase is considered identical to the pancreatic enzyme and is presumed to originate from

the latter tissue (22). The same properties which distinguish the leukocyte enzyme from the pancreatic one can therefore serve to differentiate "leukocyte elastase" from serum elastase. Accordingly, it would appear unlikely that PMN granule elastinolytic activity reflects pinocytosis of serum proteins by circulating leukocytes followed by storage of the serum enzyme within the lysosomes of these cells.

For the present, therefore, we have assumed that human PMN lysosomes contain an elastinolytic factor which is independent of that found in serum and pancreatic tissue. It remains to be determined whether this PMN-elastinolytic agent actually participates in vivo in elastolysis associated with acute arteritis. However, the following considerations at least favor this view. In those forms of arteritis thought to be associated with an immune pathogenesis, PMN may be attracted to the internal elastic lamina of the artery wall following subendothelial deposition of chemotactically-active, circulating immune complexes (23). High concentrations of PMN granule–elastase can then be released in the immediate vicinity of the elastica components in the underlying tunica media. Release of PMN granule proteinases into the extracellular environment has been shown to occur upon addition of immune complexes to leukocyte suspensions in vitro (3, 6). Local concentrations of "leukocyte elastase" which could develop under these conditions might rapidly overcome the effect of serum elastase inhibitor, a process which would be facilitated by the relatively low sensitivity of the PMN enzyme to this latter agent. (Serum elastase might also take part in the pathological elastolysis reaction, if serum elastase inhibitor became sufficiently diluted in inflammatory exudate fluids. However, under these conditions, the leukocyte enzyme by virtue of its higher threshhold of inhibition would be activated much sooner.) Other properties of the inflamed arterial tissue, such as slightly acid pH values and physiological concentrations of ions, would further favor the action of "leukocyte-elastase" over that of the serum enzyme.

The possible role of PMN lysosomal acid–cathepsins in pathological elastolysis reactions also requires consideration. Thomas (24) reported partial degradation of cartilage elastic fibers after treatment with the acid-protease, papain; while Hall (25) showed that pepsin could gradually solubilize elastin in vitro. However, the digestion of elastin by these acid-proteases was extremely slow in comparison with digestion by bacterial or pancreatic elastases, or by human leukocyte extracts in our studies. Elastase has thus been recognized as the chief proteinase capable of carrying solid elastin into solution at rates approaching those normally associated with specific enzyme-substrate interactions. Furthermore, the pH present in blood vessel walls, even in the presence of inflammatory response, is probably sufficiently stabilized by the passing plasma stream as to be close to the neutral range. The "leukocyte elastase" is still capable of moderate activity at pH 6.0–7.0, while the pH required for leukocyte

acid-cathepsins to act is far lower. Therefore, the detection of an elastinolytic factor in human PMN granules, with the ability to retain appreciable activity in the presence of physiological concentrations of salts, neutral to slightly acid pH, and moderate titers of serum elastase inhibitor, provides an attractive mechanism to account for PMN-mediated elastolysis during acute arteritis. A slow attack upon elastin by the acid-cathepsins of neutrophils may play a supplementary role in this process.

In conclusion, at least two pathologically important neutral-proteinases have now been found in human PMN granules: a collagenase (13) capable of rupturing basement membranes leading to hemorrhagic vasculitis, and an elastinolytic agent which may attack elastic lamina during acute arteritis. The complementary actions of these two enzymes upon the two major supportive constituents of connective tissues (collagen and elastin), could account for a major part of connective tissue destruction associated with acute inflammatory disease.

SUMMARY

The present study demonstrates that a granule fraction derived from human polymorphonuclear leukocytes possesses elastinolytic activity, and that the latter can be separated from the collagenase present in these cells. Properties of the human leukocyte elastase differ sufficiently from those of pancreatic elastases of different species as to suggest that the former enzyme is a distinct and separate entity. Thus, soybean trypsin inhibitor and salivary kallikrein inhibitor (Trasylol) fail to inhibit elastolysis by the pancreatic enzyme, but do inhibit the leukocyte elastinolytic agent. Elastolysis by human leukocyte granule extract does not show significant salt inhibition, whereas that catalyzed by pancreatic elastase is markedly reduced when ionic strength is increased to physiological levels. The leukocyte granule extract is at least 10 times more resistant to serum elastase inhibitor than is the purified pancreatic enzyme. Both enzymes show optimal elastolysis above pH 8.5, but the leukocyte factor still retains 50% of its maximal elastolytic activity at pH 6–7; whereas the activity of the pancreatic enzyme falls to 10% or less of its maximal value under the same conditions. The foregoing characteristics of the human leukocyte elastase suggest that it, rather than pancreatic (serum) elastase, may mediate pathological elastolysis during acute arteritis in man. In keeping with this suggestion, the present experiments also show that elastica staining of human arterial vessels is reduced by incubation of tissues with human leukocyte granule extracts in vitro.

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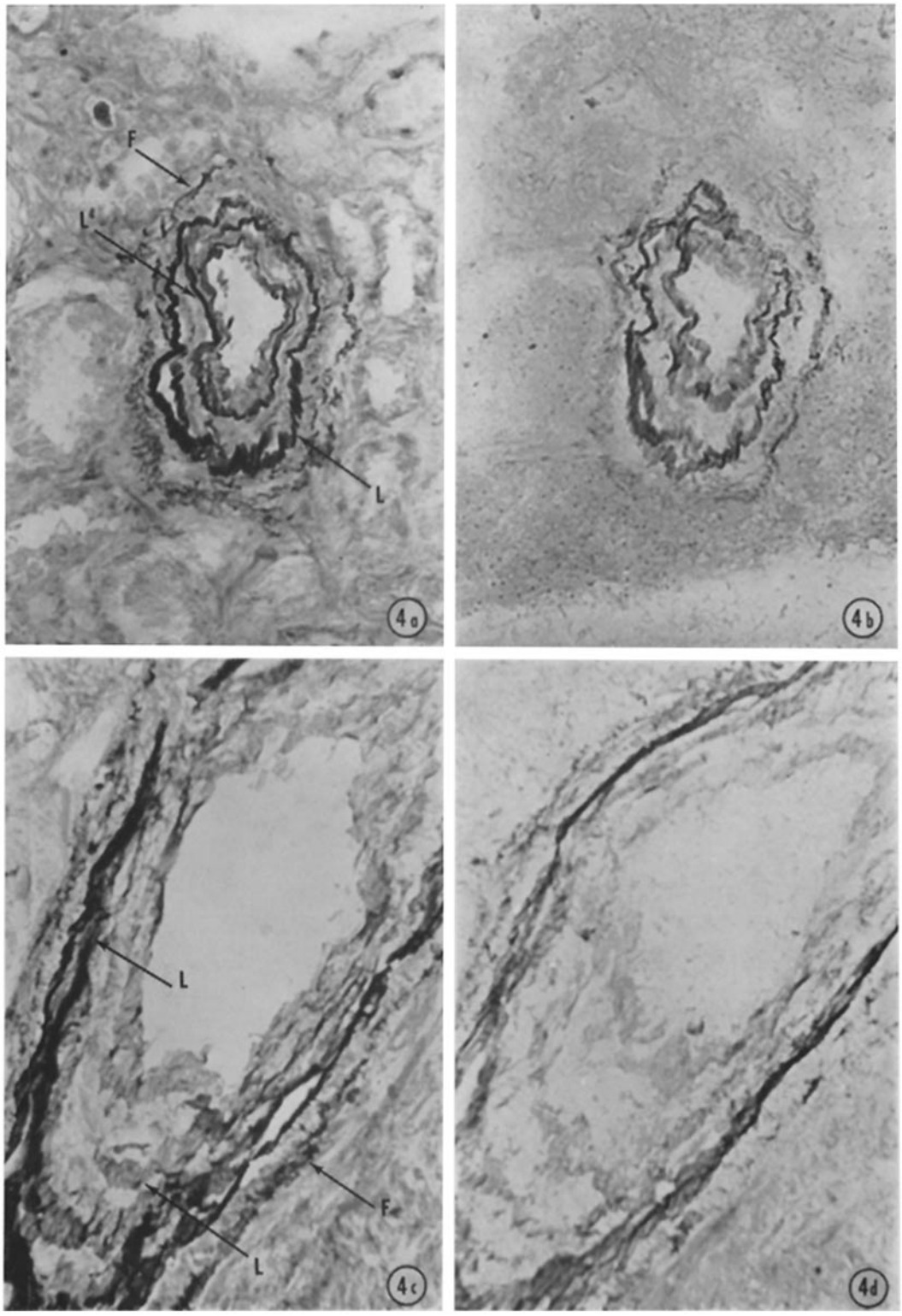
FIGS. 4 *a*–4 *d*. Effect of PMN granule extracts on the elastica staining of arteries in vitro. Vessels in frozen sections of human kidney.

FIG. 4 *a*. Control vessel after 6 hr of incubation in normal saline containing 0.01 M sodium phosphate buffer (pH 7.4 at 37°C). *L*, elastic lamellae; *F*, elastic fibers. Weigert's elastic tissue stain, × 215.

FIG. 4 *b*. Identical vessel in adjacent tissue section. Note decreased elastica staining after 6 hr of incubation in phosphate saline containing 400 μg/ml of human PMN granule protein. Weigert's elastic tissue stain, × 215.

FIG. 4 *c*. Portion of a different control vessel after incubation as in Fig. 4*a*. Weigert's elastic tissue stain, × 215.

FIG. 4 *d*. Identical vessel in adjacent tissue section, showing decreased elastica staining after incubation as in Fig. 4 *b*. Weigert's elastic tissue stain, × 215.


F
L'
L
4a
4b
L
F
L
4c
4d

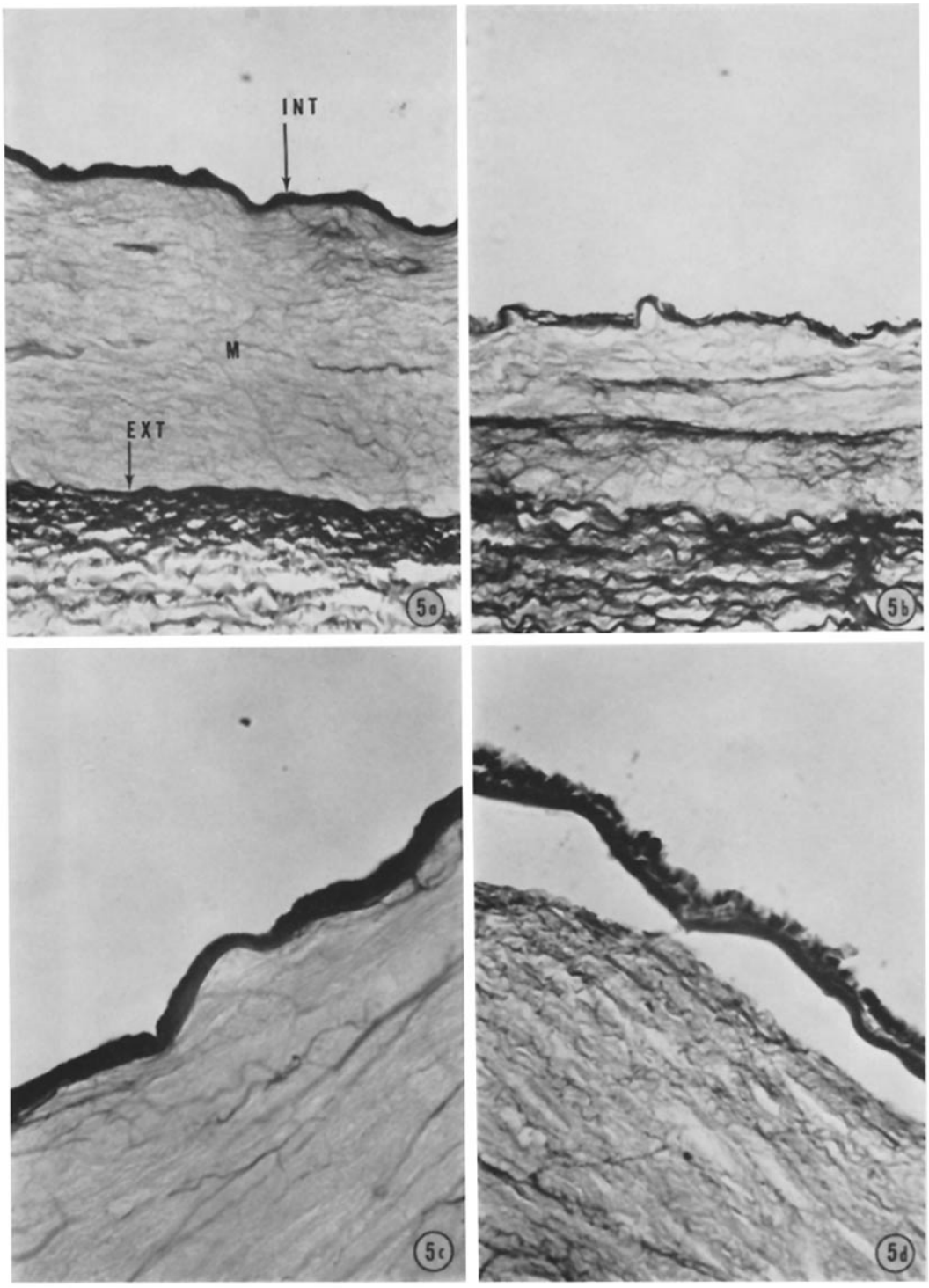
FIGS. 5 *a*–5 *d*. Effect of PMN granule extracts on the elastica staining of arteries in vitro. Human renal artery.

FIG. 5 *a*. Control segment of renal artery after 1½ hr of incubation in 0.075 M tricine buffer (pH 7.8 at 37°C). *INT*, elastica interna, *M*, tunica media, and *EXT*, elastica externa. Paraffin section, Weigert's elastic tissue stain, × 440.

FIG. 5 *b*. A different segment of the same renal artery showing fragmentation of the elastica interna after 1½ hr incubation in tricine buffer containing 450 μg/ml of human PMN granule protein. Paraffin section, Weigert's elastic tissue stain, × 440.

FIG. 5 *c*. Higher magnification of elastica interna of control arterial segment. × 1760.

FIG. 5 *d*. Higher magnification of elastica interna of arterial segment incubated with granule extract. Note detachment of elastica interna from the underlying tunica media, as well as fragmentation of the elastic membrane. × 1760.


INT
M
EXT
5a
5b
5c
5d