Depletion of Cartilage Matrix by a Neutral Protease Fraction of Human Leukocyte Lysosomes[1] (35040)

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Our laboratory is engaged in a study of the proteases of human polymorphonuclear neutrophils (PMN) and the components of connective tissue attacked by such enzymes. We previously reported that a partly purified, proteinase fraction of human PMN granules, which is active at neutral pH, degrades vascular basement membrane (1, 2) and elastin (2, 3). Lazarus *et al.* have prepared a second fraction of human PMN granules (4, 5) with collagen-monomer cleaving activity. The latter enzyme is clearly different from the elastin-degrading fraction studied by us (2).

Elastinolytic proteases are known to degrade a wide variety of proteins (6). We therefore tested the elastase-active fraction of human leukocyte granules upon other connective tissue constituents besides elastin. The present report shows that the leukocyte fraction attacks cartilage matrix protein-polysaccharide. Preliminary results also suggest that the leukocyte elastase, or a related enzyme, may be present in some pathologic joint fluids.

Materials and Methods. Incubation of cartilage. Our procedure for obtaining an elastin-degrading fraction of human PMN-granules has been described elsewhere (2). Slices of cartilage were scraped with a scalpel blade from the proximal, articular surface of the tibia of freshly killed rabbits. Tissues were incubated for 4 hr at 37° in 0.05 *M* 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl, containing 0.1 *M* NaCl and 0.001 *M* $CaCl_2$, adjusted to pH 7.4 at 37°. Some pieces of cartilage were incubated with leukocyte fractions, while others were treated with trypsin (EC 3.4.4.4) or electrophoretically purified pancreatic elastase (EC 3.4.4.7) obtained from Worthington Biochemical Corp., Freehold, N.J. Cartilage incubated alone was employed as a control for spontaneous loss of matrix protein polysaccharide. After incubation, the cartilage was fixed in 10% formalin and processed histologically for metachromatic staining of ground substance (7). Duplicate 0.1-ml aliquots of the supernatant fluids were assayed for solubilized protein using Lowry's method (8) with crystalline bovine serum albumin (Pentex Inc., Kankakee, Ill.) as reference standard.

Synovial fluid analysis. Pathologic human synovial fluids were obtained after knee taps performed for diagnostic or therapeutic purposes. Each joint fluid sample was examined for total leukocyte count, differential leukocyte count, and for esterolytic activity against t-butyloxycarbonyl-l-alanine *p*-nitrophenyl ester (NBA) obtained from Cyclo Chemical Corp., Los Angeles, Calif. The latter is a highly sensitive substrate for elastolytic enzymes (9). We previously showed that the PMN elastolytic fraction actively hydrolyzed this ester (10). The assay method for NBA esterolytic activity was the same as previously described (9, 10) except that corrections were made for changes in absorbance of synovial fluid pigments and spontaneous breakdown of substrate. Neither effect was significant and the data obtained represent true NBA hydrolysis by synovial fluid.

Results. Cartilage incubation experiments. Figure 1 shows the appearance of cartilage matrix stained with toluidine blue. It can be seen that the fraction of human PMN gran-

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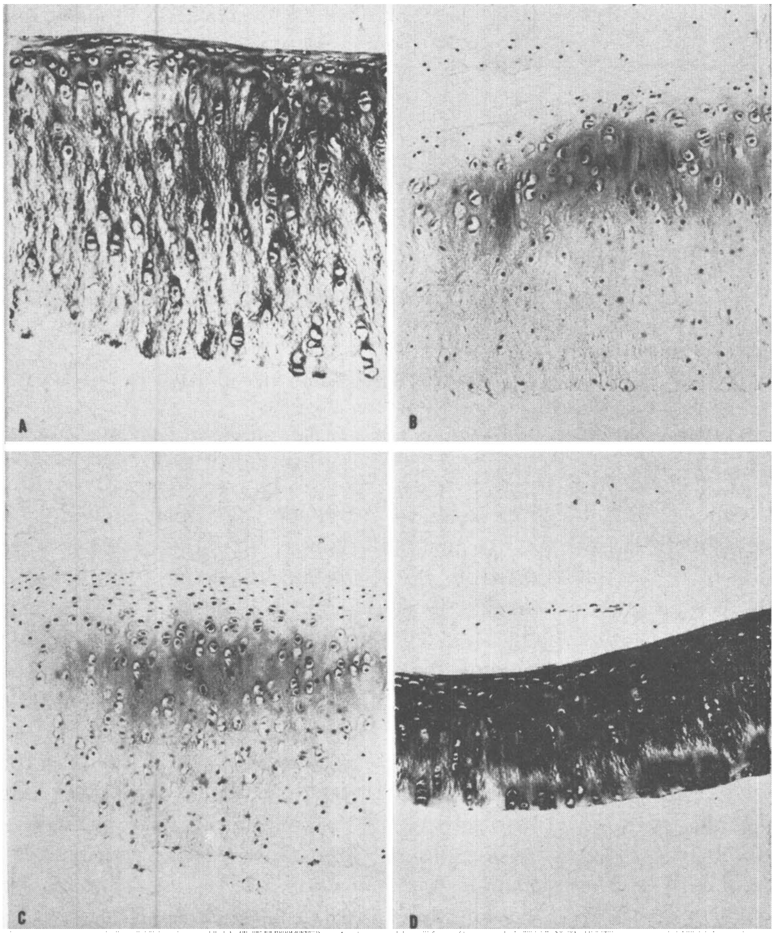

FIG. 1. Appearance of rabbit cartilage after metachromatic staining with toluidine blue. Tissue obtained from proximal articular surface of tibia. (×160). A. Control cartilage incubated 4 hr in buffer alone at pH 7.4. B. Cartilage incubated with 40 μg of trypsin. C. Cartilage incubated with 40 μg of an elastin-degrading, NBA-hydrolyzing fraction of human PMN granules. D. Cartilage incubated with 40 μg of a granule fraction containing collagen-degrading activity alone (see text).

ules containing elastinolytic and NBA-esterolytic activity was active in depleting cartilage ground substance of metachromatically-stained material (Fig 1C). This result could be correlated with measurements of protein released into the incubation medium (Table I). The active PMN granule fraction was about 65% as effective in solubilizing matrix protein-polysaccharide as was an equal weight of crystalline trypsin. Pancreatic elastase (electrophoretically purified) also degraded cartilage matrix in these experiments. This was evident both from results of metachromatic staining and from measurement of protein release. Concentrations of the PMN granule fraction and of pure elastase which gave equivalent NBA hydrolysis released equivalent amounts of protein from cartilage matrix (see Table I). A second PMN fraction, prepared according to methods reported for purification of the collagen monomer-cleaving enzyme (4, 5), proved relatively inactive on cartilage matrix (Fig. 1D and Table I). This same fraction, however, was capable of solubilizing native, beef-tendon collagen (2).

NBA-esterolysis by pathologic joint fluids. Samples of knee joint synovial fluids were

TABLE I. Release of Protein from Cartilage Slices by Human PMN Granule Fractions and Pure Enzymes.[a]

Fraction	NBA hydrolysis rate[b]	Protein released (μg/10 mg cartilage)
Buffer alone	—	220 (200–260)
Trypsin (40 μg)	0.005	759 (730–800)
Pancreatic elastase (40 μg)	0.100	580 (560–600)
PMN elastin-degrading fraction (40 μg)	0.100	568 (425–680)
PMN collagen monomer-cleaving fraction (40 μg)	0.000	244 (220–277)

[a] Incubations at pH 7.4, 4 hr, 37°. Values shown are averages of five separate experiments (ranges given in parentheses).

[b] Increase in A 347.5 nanometers/30 sec due to liberated *p*-nitrophenol (NBA = t-butyloxycarbonyl-l-alanine *p*-nitrophenyl ester).

obtained from 12 patients with arthritides of varying etiology. Synovial fluid was received from one individual (TM) on three separate occasions, one time from both knees, raising the total number of samples tested to 15. Significant levels of NBA esterase activity (change in absorbance/min/0.1 ml $\gtreqless$ 0.005) were detected in about half the joint fluid samples. However, the relationship between numbers of leukocytes in the joint fluid and NBA-esterase activity of the samples was highly variable. Additional factors (such as drugs administered) may have contributed to this variability. Figure 2 plots rate of hydrolysis of NBA against concentration of joint fluid assayed (sample CF). It can be seen that the relationship was linear over the range of concentrations tested. This same joint fluid, heated to 100° for 10 min, was found to have lost all of its NBA-esterase activity. The pH of the synovial samples exposed to ambient air resembed that of plasma measured under the same conditions (see Table II), suggesting that neutral proteases could be active in the joint fluids *in vivo*.

Discussion. The present experiments show that a fraction of human PMN granules with neutral-proteinase activity against elastin and esterase activity against the elastase substrate NBA, is capable of releasing protein from rabbit articular cartilage and of causing loss of matrix metachromasia. In addition, half of a series of pathologic joint fluids were found to contain readily detectable levels of a thermolabile, NBA-hydrolyzing agent. There is no evidence, however, that this agent

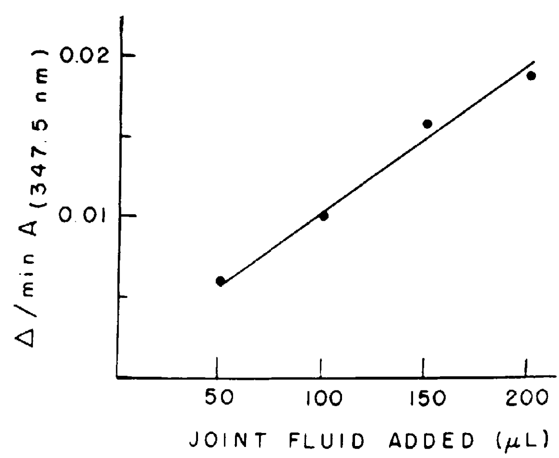

FIG. 2. Effect of joint fluid concentration on NBA hydrolysis. Ordinate: Increase in absorbance/min due to liberated *p*-nitrophenol. Abscissa: Microliters of joint fluid added per 3.0 ml of 0.2 m*M* NBA.

TABLE II. NBA Hydrolysis by Pathologic Synovial Fluids.

Patient	Age (yr)	Sex	Diagn.[a]	WBC (no./mm^3)	PMN (%)	Δ^A/min 347.5 nm[b]	Sample pH
AR	49	M	RA	15,000	90	.008	7.7
TM	50	M	RA	150	nt	.002	nt
JH	49	F	RA	100	66	.004	nt
WE	64	M	RA	200	53	.003	nt
GF	28	F	RA	20,000	83	.004	nt
ER	57	F	OA	400	nt	.006	nt
TR	72	M	GA	5000	53	.007	7.9
TM	50	M	RA	100	nt	.002	nt
CF	53	F	RA	4000	53	.010	nt
AY	68	F	OA	300	nt	.002	8.0
TM-R	50	M	RA	2000	nt	.002	7.3
TM-L	50	M	RA	100	nt	.002	nt
CG	74	M	RA	2000	nt	.005	7.4
EG	74	F	RA	10,000	78	.006	7.4
JD	60	F	RA	4000	45	.006	nt

[a] RA = rheumatoid arthritis; OA = osteoarthritis; GA = gouty arthritis.

[b] Change in absorbance at 347.5 nm/min/0.1 ml joint fluid. (All samples were centrifuged and enzyme assays performed on the cell-free supernatants.) nt = not tested.

originated from leukocytes present in the joint space. An alternate possibility, not yet tested, is that synovial fluid NBA-esterase activity is due to serum elastase.

Whole lysates of human peripheral blood PMN have been reported to degrade bovine nasal septum chondromucoprotein (11). It seems reasonable to suggest that a granule neutral protease such as that described herein is responsible for the protein-polysaccharide degrading activity of these whole cell lysates. Furthermore, a neutral-acting protein-polysaccharidase has been found in rabbit PMN granules by Weissmann and Spilberg (12), and more recently, an NBA-hydrolyzing enzyme was also detected in such granule preparations (13). It is tempting to suggest that the NBA-esterase is also responsible for cartilage degradation by rabbit PMN. However, other neutral proteases may be present in PMN lysosomes, and it is not presently established that the elastinolytic enzyme is solely responsible for breakdown of matrix proteoglycan. This latter possibility receives some support from the known broad substrate affinities of elastases and from our demonstration that pure pancreatic elastase attacks cartilage matrix.

Clearly, synovial lining tissue and pannus-formation represent further sources of potentially injurious enzymes in joint disease, in addition to exudative PMN. Furthermore, it should be obvious that the present data afford no information regarding the effectiveness of PMN granule proteases upon cartilage *in vivo*, where interference by protease inhibitors of the synovial fluid (14, 15) might take place. Nevertheless, our results do show cartilage matrix-degrading activity in human PMN granules, specifically in that granule fraction previously found to attack arterial elastin. These findings suggest that a PMN elastase could play a broad role in tissue damage associated with acute inflammation, perhaps including inflammatory diseases of the joints. The observation of NBA-esterolytic activity in rheumatoid joint fluid lends further support to this view.

Summary. A neutral protease fraction of human PMN granules which degrades elastin and hydrolyzes l-alanine *p*-nitrophenyl ester (NBA) was tested on rabbit articular cartilage. Forty micrograms of this fraction released 568 μg of protein from 10 mg of cartilage in 4 hr (average value of five experiments). This effect was correlated with a nearly complete loss of metachromatic staining of the cartilage ground substance. Electro-

phoretically purified pancreatic elastase also degraded cartilage matrix, supporting the suggestion that the elastinolytic protease of the PMN granule fraction was primarily responsible for its attack upon matrix proteoglycan. Another fraction of the granules, containing only collagenolytic activity but no NBA-esterolytic activity, gave insignificant matrix degradation. Of 15 human, pathologic synovial fluid samples, seven contained readily measurable NBA-esterase activity.

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