

## **Degradation of cartilage proteoglycan by human leukocyte granule neutral proteases--a model of joint injury. I. Penetration of enzyme into rabbit articular cartilage and release of <sup>35</sup>SO<sub>4</sub>-labeled material from the tissue.**

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### **Research Article**

The present work was undertaken to explore the effect of two purified neutral proteases derived from human peripheral blood polymorphonuclear leukocytes (PMN) on articular cartilage as a model of joint injury. Human leukocyte elastase and chymotrypsin-like enzyme, purified by affinity chromatography, released <sup>32</sup>SO<sub>4</sub> from labeled rabbit articular cartilage slices in vitro. Release of isotope was initially delayed, suggesting that either a lag in enzyme penetration occurs or that size of degradation fragments is a limiting factor in diffusion of label out of the tissue. The release of <sup>35</sup>SO<sub>4</sub> was inhibited by preincubation of elastase and chymotrypsin-like enzyme with human alpha 1-anti-trypsin, or with their specific chloromethyl ketone inactivators, and the action of elastase was also inhibited by a monospecific antiserum to PMN elastase, freed of major serum proteinase inhibitors. Immunohistochemical staining procedures revealed the presence of PMN elastase inside the matrix of cartilage slices after a 20-min exposure of tissue to either the pure enzyme or crude PMN granule extract. Serum alpha 1-antitrypsin failed to penetrate into the cartilage slices under identical in vitro conditions. In association with the results reported in the accompanying paper, these findings suggest a model of cartilage matrix degradation by PMN neutral proteases in which local protease-antiprotease imbalance, coupled with different rates of penetration of protease and antiprotease into target tissue, plays a key role [...]

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# Degradation of Cartilage Proteoglycan by Human Leukocyte Granule Neutral Proteases—A Model of Joint Injury

## I. PENETRATION OF ENZYME INTO RABBIT ARTICULAR CARTILAGE AND RELEASE OF $^{35}\text{SO}_4$ -LABELED MATERIAL FROM THE TISSUE

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**ABSTRACT** The present work was undertaken to explore the effect of two purified neutral proteases derived from human peripheral blood polymorphonuclear leukocytes (PMN) on articular cartilage as a model of joint injury. Human leukocyte elastase and chymotrypsin-like enzyme, purified by affinity chromatography, released  $^{35}\text{SO}_4$  from labeled rabbit articular cartilage slices in vitro. Release of isotope was initially delayed, suggesting that either a lag in enzyme penetration occurs or that size of degradation fragments is a limiting factor in diffusion of label out of the tissue. The release of  $^{35}\text{SO}_4$  was inhibited by preincubation of elastase and chymotrypsin-like enzyme with human alpha 1-antitrypsin, or with their specific chloromethyl ketone inactivators, and the action of elastase was also inhibited by a monospecific antiserum to PMN elastase, freed of major serum proteinase inhibitors. Immunohistochemical staining procedures revealed the presence of PMN elastase inside the matrix of cartilage slices after a 20-min exposure of tissue to either the pure enzyme or crude PMN granule extract. Serum alpha 1-antitrypsin failed to penetrate into the cartilage slices under identical in vitro conditions. In association with the results reported in the accompanying paper, these findings suggest a model of cartilage matrix degradation by PMN neutral proteases in which local protease-antiprotease imbalance, coupled with different rates of penetration of protease and antiprotease into target tissue, plays a key role in accounting for matrix damage.

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## INTRODUCTION

Lysosomes of human peripheral blood polymorphonuclear leukocytes (PMN)<sup>1</sup> contain several proteolytic enzymes that, uncharacteristically for lysosomal enzymes, have pH optima close to the physiological range (1, 2). These enzymes have attracted particular attention as pathogenetic determinants in connective tissue injury associated with inflammation (3, 4). One type of injury in which such enzymes may play a significant role is cartilage destruction during arthritis (5). The pH of synovial fluid in various joint diseases is generally near neutrality (6.6–7.4) (6). Furthermore, crude extracts of human PMN cytoplasmic granules (G) and neutral protease-enriched fractions isolated from these granules can degrade cartilage matrix proteoglycans in tissue slices at neutral pH (7–10). More recently, at least three neutral proteases have been identified and purified from human PMN: a collagenase, an elastase (E), and a chymotrypsin-like enzyme (CT). By virtue of their properties, these enzymes should be capable of attacking joint connective tissues, including the proteoglycans of cartilage matrix (for a review, see ref. 11).

In this paper, we report our observations on the effect

<sup>1</sup> *Abbreviations used in this paper:* AAPACK, *N*-acetyl-L-alanyl-L-alanyl-L-prolyl-L-alanine chloromethyl ketone; CT, purified PMN chymotrypsin-like enzyme; E, purified PMN elastase; FITC-GARG, fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin antiserum; G, whole (crude) extract of PMN granules; HRP-GARG, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antiserum; PBS, 0.01 M sodium phosphate plus 0.15 M NaCl, pH 7.0; PMN, polymorphonuclear leukocytes; ZPCK, *N*-carbobenzoxyl-L-phenylalanine chloromethyl ketone.

of two of these enzymes, E and CT, in highly purified form, on cartilage. In the accompanying paper, the effect of these same enzymes on isolated proteoglycans derived from cartilage is described. For the studies with whole tissue, we employed the technique of monitoring  $^{35}\text{SO}_4$  release from prelabeled rabbit articular cartilage, as this method has been used by others (9, 10) in similar studies. The vast bulk of administered  $^{35}\text{SO}_4$  that enters hyaline cartilage becomes incorporated into the proteoglycans thereof (12, 13). Our results will show that highly purified E or CT, separated from other granule components by affinity chromatography, releases  $^{35}\text{SO}_4$ -containing substances from rabbit articular cartilage slices. In addition, the kinetics of isotope release and the effects upon release of specific chloromethyl ketone proteinase inactivators, monospecific anti-elastase antiserum, and the endogenous serum proteinase inhibitor, alpha 1-antitrypsin, will be described. Also, the ability of one of these PMN enzymes (E), and the inability of its principal serum inhibitor (alpha 1-antitrypsin) to penetrate into cartilage slices *in vitro*, as studied by immunohistochemical techniques, will be reported. The degradation of isolated cartilage proteoglycans by each of the purified leukocyte enzymes and the partial characterization of the recovered digestion products will be the subjects of a second paper (14).

## METHODS

**Materials.**  $^{35}\text{SO}_4$ , as  $\text{Na}_2^{35}\text{SO}_4$ , was obtained from New England Nuclear, Inc., Boston, Mass. (sp act, 773 mCi/mM). New Zealand albino rabbits were from Hare Rabbits, Inc., Hewitt, N. J. Human blood for preparation of leukocyte enzymes was purchased from Inter-County Blood Services, Rockville Centre, N. Y., a division of the New York Blood Center. *N*-acetyl-L-alanyl-L-alanyl-L-prolyl-L-alanine chloromethyl ketone (AAPACK) and *N*-carbobenzoxyl-L-phenylalanine chloromethyl ketone (ZPCK) were generously provided through a contract from the Division of Lung Diseases, National Institutes of Health, by Drs. J. Powers and P. M. Tuhy of the Georgia Institute of Technology, Atlanta, Ga. Human alpha 1-antitrypsin was from Worthington Biochemical Corp., Freehold, N. J., and rabbit antiserum to human alpha 1-antitrypsin was purchased from Behring Diagnostics, American Hoechst Corp., Somerville, N. J. 1 ml of this antiserum precipitated 0.55 mg of human alpha 1-antitrypsin. Fluorescein-isothiocyanate-conjugated goat anti-rabbit immunoglobulin antiserum (FITC-GARG) was from Meloy Laboratories Inc., Springfield, Va. This antiserum had an antibody protein concentration of 9.0 mg/ml and contained 3.0  $\mu\text{g}$  fluorescein/mg antibody protein. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antiserum (HRP-GARG) was obtained from Cappel Laboratories, Inc., Downingtown, Pa. The antibody protein concentration of this antiserum was 4.1 mg/ml.

**Preparation of  $^{35}\text{SO}_4$ -labeled rabbit articular cartilage.** Rabbits were injected intravenously with  $\text{Na}_2^{35}\text{SO}_4$  (750  $\mu\text{Ci/kg}$ ) in physiological saline and sacrificed 18 h later with pentobarbital. The proximal humeri and tibiae and the proximal and distal femora were removed and slices of

articular cartilage were then dissected from the joints in the cold and subsequently stored at  $-80^\circ\text{C}$ . Further details of these procedures were described earlier (15).

**Preparation of purified PMN enzymes.** Leukocytes (85–95% PMN) were harvested from human peripheral blood, and the cytoplasmic granule fraction was isolated from the cells by differential centrifugation of leukocyte homogenates prepared in 0.34 M sucrose, according to previously described techniques (16). Differential salt extraction of the granules and isolation of E and CT, with affinity chromatography on 4-phenylbutylamine-Affi-Gel (Bio-Rad Laboratories, Richmond, Calif.), were carried out as has been previously reported (17, 18).

**Assay of  $^{35}\text{SO}_4$ -release.** 3–4 mg (wet wt) of cartilage substrate was cut into pieces measuring 1–2 mm on edge, containing about 1,500 cpm each, which were incubated for different time intervals up to 0.5 h in siliconized 5-ml glass ampoules at  $37^\circ\text{C}$ . Incubations were carried out in 1 ml of 0.05 M phosphate buffer (pH 7.5) + 0.3 M NaCl, and 1–3  $\mu\text{g}$  of purified enzyme protein was used per milligram tissue. The high ionic strength was required to facilitate solubility of CT. At the end of the desired incubation intervals, the ampoules were rapidly transferred to an ice bath and 0.5-ml samples of incubation medium were removed and mixed with 10 ml of 2.0% Liquifluor (Pilot Chemicals, Inc., Watertown, Mass.) in 1:1 (vol/vol) toluene:ethylene glycol monomethyl ether (ACS grade). These samples were subsequently counted for radioactivity. The remainder of the incubation medium was immediately removed from all vessels, the cartilage briefly rinsed with ice cold 0.1 M acetate buffer (pH 4.0), and the tissue then hydrolyzed at  $110^\circ\text{C}$  overnight in 8 M HCl. Portions of hydrolyzate were diluted with water and lyophilized to eliminate HCl. Lyophilates were redissolved in water, mixed with 10 ml of the liquid scintillation mixture described above, and counted for radioactivity by liquid scintillation spectrometry. The fraction of radioactivity released into the medium and the fraction remaining in the cartilage at the end of each incubation interval were determined from the radioactivity values, and the released fraction was expressed as percent of total radioactivity initially present in the tissue sample. All samples were tested in duplicate or triplicate.

**Inactivators and inhibitors.** In selected experiments, the purified PMN enzyme preparations were preincubated with 0.2 mM solutions of AAPACK or ZPCK for 1 h at  $20^\circ\text{C}$  before they were added to the cartilage substrate. Both inactivators were dissolved in the phosphate-buffered saline described above containing 20% dimethylsulfoxide. For these experiments, control enzymes were preincubated in the same buffer solution as was used to dissolve the inactivators; 20% dimethylsulfoxide did not affect activity of either enzyme.

In other tests, the purified leukocyte enzymes were preincubated for 10 min with 500  $\mu\text{g/ml}$  of human alpha 1-antitrypsin, and the mixture was then added to the cartilage. 1 mg of this antitrypsin preparation (Worthington lot 53J411) gave 100% inhibition of bovine trypsin (56.8  $\mu\text{g}$ ) after 10 min of incubation at  $22^\circ\text{C}$  (benzoyl-arginine-*p*-nitroanilide assay). Previous experience (18) showed that this same preparation of human alpha 1-antitrypsin could significantly inhibit the activity of both PMN enzymes against their respective low mol wt synthetic substrates.

In another experiment, E was preincubated for 15 min before addition to cartilage with a monospecific rabbit anti-E antiserum (described below) first rendered free of rabbit alpha 1-antitrypsin and alpha 2-macroglobulin proteinase inhibitors. The concentration of antiserum protein in the pre-

incubation mixture was 9.0 mg/ml, and the preincubation medium was the same phosphate-saline buffer described above.

**Monospecific anti-elastase antiserum.** The rabbit antiserum to E that we used in these experiments gave a single precipitin line of identity when reacted against E and G in double immunodiffusion tests, but it failed to react against any other component of G. It was also found to be monospecific for E when tested against serial extracts of acrylamide gel electrophoretograms of G. The preparation and characterization of this antiserum has been reported in greater detail elsewhere (17). The same antiserum was used for two different purposes in these experiments. It was employed in the study of inhibition by antibodies of cartilage matrix attack by E, for which it was first rendered free of trypsin-inhibitor activity (alpha 1-antitrypsin and alpha 2-macroglobulin) by passage through a trypsin-Sepharose column as described elsewhere.<sup>2</sup> The antiserum was also used for visualization of E penetration into cartilage slices by indirect immunofluorescence and related immunohistochemical techniques. For the latter purpose, whole, untreated antiserum was employed.

**Immunohistochemical procedures.** For study of the penetration of E and serum alpha 1-antitrypsin into cartilage in vitro, tissue slices were cut at about the same thickness, but at twice the length and width as those used for the study of radiolabeled release, and these pieces of tissue were then incubated according to one of the following protocols: (A) in buffer alone for 30 min; (B) in buffer alone for 10 min before addition of E to a final concentration of 70  $\mu$ g purified enzyme protein/ml and continued incubation for 20 additional min; (C) as in (B), except that G was substituted for E to give 600  $\mu$ g granular proteins/ml in the incubation medium during the final 20 min; (D) in buffer alone for 10 min before addition of fresh human serum to a final concentration of 27% (vol/vol) and continued incubation for 20 additional min. In all instances, the buffer used was 0.05 M sodium phosphate, pH 7.5, in 0.3 M NaCl, and the incubation temperature was 37°C. Higher concentrations of enzyme were employed in these experiments than in the radiolabeled-release experiments, to provide optimal conditions for visualization of enzyme in the tissue by immunohistochemistry.

At the end of the incubation, the tissue slices were removed from the solutions and either rinsed briefly in fresh buffer or else simply drained without further washing (when surface-adherent enzyme or serum proteins were desired as a positive staining control). These tissues were then immediately frozen in iso-pentane chilled by liquid nitrogen to -170°C. Thereafter, the frozen tissue was sectioned at 6  $\mu$ m thickness in a cryostat. The sections were transferred to glass slides, air-dried for a minimum of 1 h at 35°C and then fixed directly by one of three methods: (A) immersion in absolute acetone (4°C) for 40 s, or (B) for 10 min, or (C) as in (B), before 20 min exposure to cold, 10% neutral-buffered formaldehyde solution. No effect of varying fixatives was observed on immunohistochemically detectable E or alpha 1-antitrypsin in the tissues. It should be emphasized that the tissue sections were never washed before fixation. The reasons for this will be given below. After fixation, the sections were washed three times with mild agitation for 5 min each time in normal saline

<sup>2</sup>Feinstein, G., and A. Janoff. 1975. The use of polyacrylamide gel electrophoresis to quantitate activity of leukocyte elastase and to detect its activity in complexes of biological interest. *Anal. Biochem.* Submitted for publication.

buffered to pH 7.0 with 0.01 M sodium phosphate (PBS). They were then incubated in a moist atmosphere, at 22°C, for 30 min in one of four different primary reagents; PBS, normal rabbit serum, anti-human E antiserum (rabbit), or anti-human alpha 1-antitrypsin antiserum (rabbit). After three additional PBS washes (10 min each), all sections were stained for 30 min at 22°C with a 1:4 dilution (in PBS) of FITC-GARG or a 1:20 dilution of HRP-GARG (see Materials). After three final PBS washes (10 min each), they were handled in one of two ways. HRP-GARG-treated sections subsequently reacted at 22°C for 10 min with a freshly prepared solution of 0.04% 3,3'-diamino-benzidine in pH 7.6 Tris-HCl buffer (0.05 M) containing 0.0012% hydrogen peroxide. Slides were post-osmicated for 10 min in 1% osmium tetroxide, dehydrated in alcohol, cleared in xylene, and mounted for bright field microscopy. FITC-GARG treated sections were directly mounted in Elvanol (E. I. duPont de Nemours & Co., Wilmington, Del.) (17% polyvinyl alcohol + 33% glycerol in PBS), covered with glass cover slips, and examined with a Zeiss Universal microscope (Carl Zeiss, Inc., New York). FITC-GARG-treated sections were observed by epi-illumination fluorescence microscopy with a quartz-halogen (100 W-12 V) light source, KP490 interference filter, FL500 reflector, and a 530 barrier filter (all Carl Zeiss, Inc.).

**Protein assay.** Protein concentrations were determined throughout these experiments by the Lowry et al assay (19), with twice-crystallized bovine serum albumin as a reference standard.

## RESULTS

**Release of <sup>35</sup>SO<sub>4</sub> from rabbit articular cartilage by purified human PMN enzymes.** Fig. 1 shows the kinetics of <sup>35</sup>SO<sub>4</sub>-release from labeled joint cartilage substrate, when the latter was incubated for 30 min with purified E or CT. The results obtained with either enzyme were virtually identical. After an initial 10-min lag, there was a rapid rise in released radioactivity, which did not level off within the remainder of the observation period.

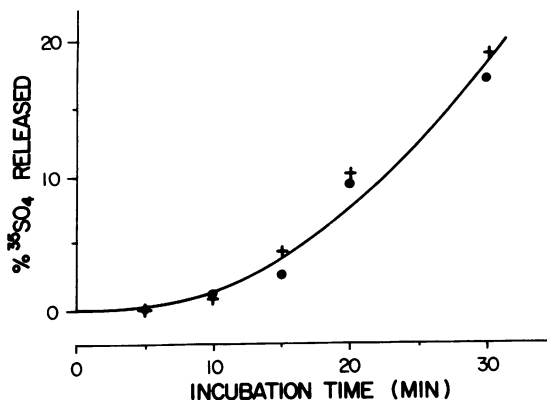


FIGURE 1 Release of radioactivity (<sup>35</sup>SO<sub>4</sub>) from rabbit articular cartilage by purified human PMN enzymes, as a function of time. ●, elastase (1  $\mu$ g/ml); +, chymotrypsin-like enzyme (1  $\mu$ g/ml). Incubations were carried out in neutral buffer (0.05 M Na phosphate, pH 7.5, containing 0.3 M NaCl).

TABLE I  
The Effect of Chloromethyl Ketone Derivatives on the Release of Radiosulfate from Rabbit Articular Cartilage by Purified Human PMN Elastase (E) and Chymotrypsin-Like Enzyme (CT)

Enzyme*	Release
	%
E	100.0†
E + AAPACK	7.6
E + ZPCK	78.3
CT	100.0†
CT + AAPACK	100.0
CT + ZPCK	6.7

\* For concentrations of enzymes and inactivators, see Methods.

† Values for percent of total tissue radiosulfate released by purified enzymes after 30 min incubation at 37°C have been normalized to 100 for convenient comparison with release given by enzymes preincubated with inactivators. Thus, the percent inhibition given by the various pretreatments = 100 - value in Table. All data were first corrected for background radiosulfate release, after 30 min incubation, from cartilage alone. (The latter rarely exceeded 3% of total tissue radioactivity).

*Inhibition of  $^{35}\text{SO}_4$  release by specific chloromethyl ketone inactivators of PMN enzymes.* In Table I, the values for  $^{35}\text{SO}_4$  released at 30 min in Fig. 1 have been corrected for background autolysis (1-3%) and then normalized to 100% for convenient comparison to  $^{35}\text{SO}_4$  release under identical conditions given by enzymes pretreated with their specific chloromethyl ketone inactivators. AAPACK was used to inhibit E (20) while ZPCK was used to inhibit CT (18). As shown in the Table, almost complete inhibition of  $^{35}\text{SO}_4$  release occurred when each enzyme was first exposed to its own

TABLE II  
The Effect of Various Protein Inhibitors on the Release of Radiosulfate from Rabbit Articular Cartilage by Purified PMN Proteases

Enzyme*	Release
	%
E	100.0†
E + human alpha 1-antitrypsin	6.1
E + monospecific antiserum§	0.0
CT	100.0†
CT + human alpha 1-antitrypsin	10.0

\* † See footnotes to Table I.

§ Rabbit antiserum to E, free of alpha 1-antitrypsin and alpha 2-macroglobulin proteinase inhibitors. This antiserum had no detectable trypsin-inhibiting capacity. (For further details, see Methods and footnote 2.)

specific inactivator, but not when it was exposed to an inactivator specific for the other enzyme.

*Inhibition of  $^{35}\text{SO}_4$  release by E after preincubation of the enzyme with a monospecific antiserum.* For this experiment, the anti-E antiserum was first rendered free of rabbit alpha 1-antitrypsin and alpha 2-macroglobulin by a column of immobilized-trypsin.<sup>2</sup> Table II shows that  $^{35}\text{SO}_4$  release by E was inhibited by preincubation of the enzyme with the absorbed antiserum. Previous work<sup>2</sup> had shown that 360 µg protein of the absorbed antiserum could complex 0.7 µg of E.

*Inhibition of  $^{35}\text{SO}_4$  release by alpha 1-antitrypsin.* Previous work has shown that human alpha 1-antitrypsin inhibits the activity of both E (18, 21) and CT (18, 22-24) when these are tested on a variety of synthetic and protein substrates. The data in Table II show that human alpha 1-antitrypsin also prevents these two PMN neutral proteases from releasing  $^{35}\text{SO}_4$  from cartilage slices. In view of the reported presence of alpha 1-antitrypsin in synovial effusions (25, 26), these observations could be relevant to our understanding of the mechanism of PMN enzyme-mediated injury to articular cartilage *in vivo*. However, since the inhibitions shown in Table II were obtained by pretreatment of the enzymes with alpha 1-antitrypsin in the absence of cartilage substrate, such experiments do not necessarily reflect the complex conditions governing interaction of protease and anti-protease in inflamed joints (see Discussion). With this in mind, we next explored the relative abilities of E and serum alpha 1-antitrypsin to penetrate into cartilage matrix.

#### Penetration of PMN elastase (E) and serum alpha 1-antitrypsin into cartilage slices

*Penetration of enzyme.* Fig. 2A and C show the results of incubation of rabbit cartilage with purified E and G, respectively, before immunohistochemical staining of the treated tissue with the anti-E antiserum and FITC-GARG (see Methods). A zone of matrix fluorescence, indicating presence of enzyme in the tissue, can be seen extending inward from the exposed surfaces. The pattern seen in 2A, showing penetration of enzyme from both the intact and the dissected surfaces of the tissue slice, was clearly observed in the majority of positive specimens. This pattern suggests that diffusion of enzyme into the tissue was not dependent on mechanical injury of the cartilage imposed during its dissection from the articular surface. Control tissue incubated in the absence of enzyme did not show matrix fluorescence when subjected to identical staining procedures. E- or G-treated tissue stained with normal rabbit serum in place of anti-E antiserum was also negative (Fig. 2B and D).

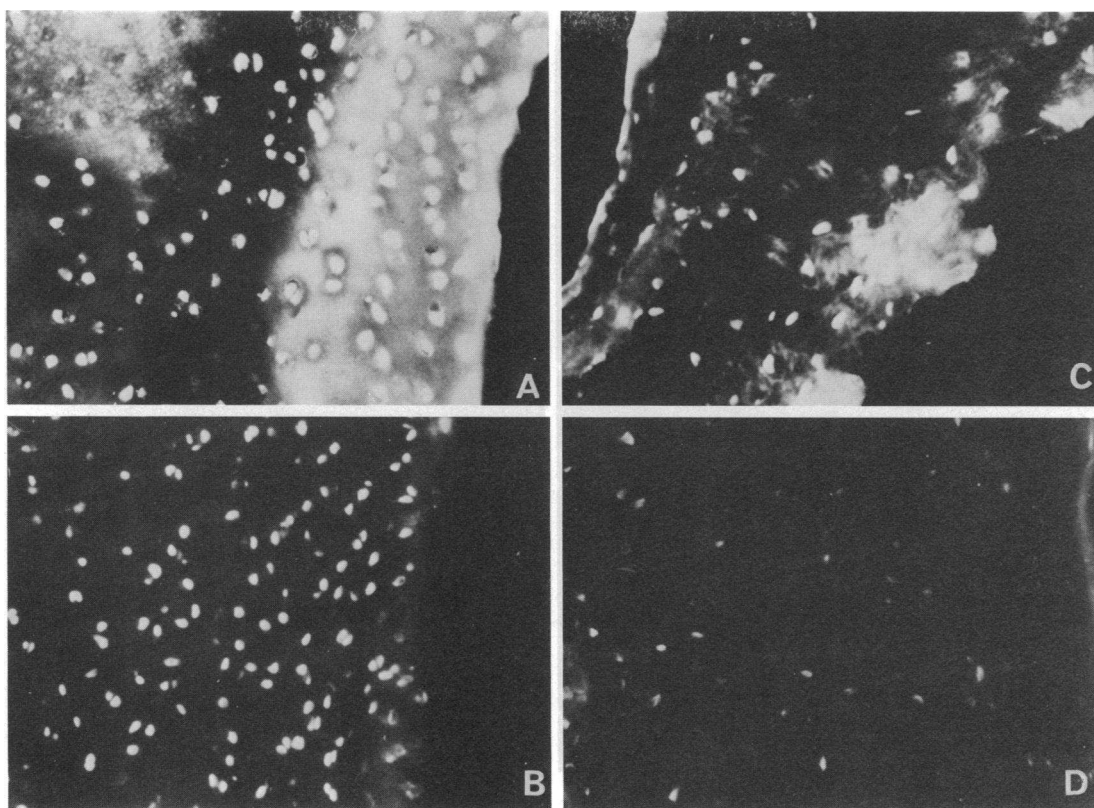


FIGURE 2 Immunohistochemical demonstration of the penetration of human PMN elastase into slices of rabbit articular cartilage (A and B, incubation of cartilage tissue with purified enzyme. C and D, incubation with crude PMN granule extract) by indirect immunofluorescence technique with fluorescein-conjugated goat anti-rabbit immunoglobulin antiserum as secondary stain. In A and C, rabbit anti-human PMN elastase antiserum was the primary stain. In B and D, normal rabbit serum was the primary stain (control). Original magnification  $\times 160$ .

As is evident in Fig. 2, fluorescence was also present in the immediate vicinity of the chondrocytes in all specimens, whether they were stained with anti-E antiserum or normal rabbit serum. Chondrocyte fluorescence did not depend on prior exposure of the tissue to enzyme, but rather was related to treatment of the tissue sections with any rabbit serum during the primary staining procedure. Sections exposed to saline in place of rabbit serum at the primary step and then stained with FITC-GARG did not show any chondrocyte fluorescence. Thus, unlike matrix fluorescence, chondrocyte fluorescence proved to be an artifact of the immunohistochemical staining procedure. This artifact may have resulted from the accumulation of rabbit gamma globulins and other serum proteins in the chondrocyte lacunae, causing an increased resistance of these proteins to removal during washing.

*Lack of penetration of alpha 1-antitrypsin.* Fig. 3 shows the results obtained when cartilage was incubated briefly in buffer and then whole fresh human serum was

added for an additional incubation interval (see Methods). Subsequent staining of sections from such tissue with rabbit antiserum to human alpha 1-antitrypsin (Fig. 3A), or with normal rabbit serum (Fig. 3B), followed by secondary staining with FITC-GARG, failed to reveal any significant penetration of the serum proteinase inhibitor into the tissue matrix.

Since alpha 1-antitrypsin is a negatively charged glycoprotein and cartilage matrix is rich in negatively charged proteoglycan complexes, retention of the inhibitor within this tissue may normally be impeded by repulsion of like charges. On the other hand, PMN elastase is a highly basic protein (27), and its retention within the same tissue could be facilitated by an ionic bridging effect. For this reason, it appeared possible that some alpha 1-antitrypsin might have penetrated into the cartilage slices, but thereafter been easily washed out of the tissue, whereas this would not have occurred in the case of ionically bound E. To minimize the possible loss of alpha 1-antitrypsin during the washing

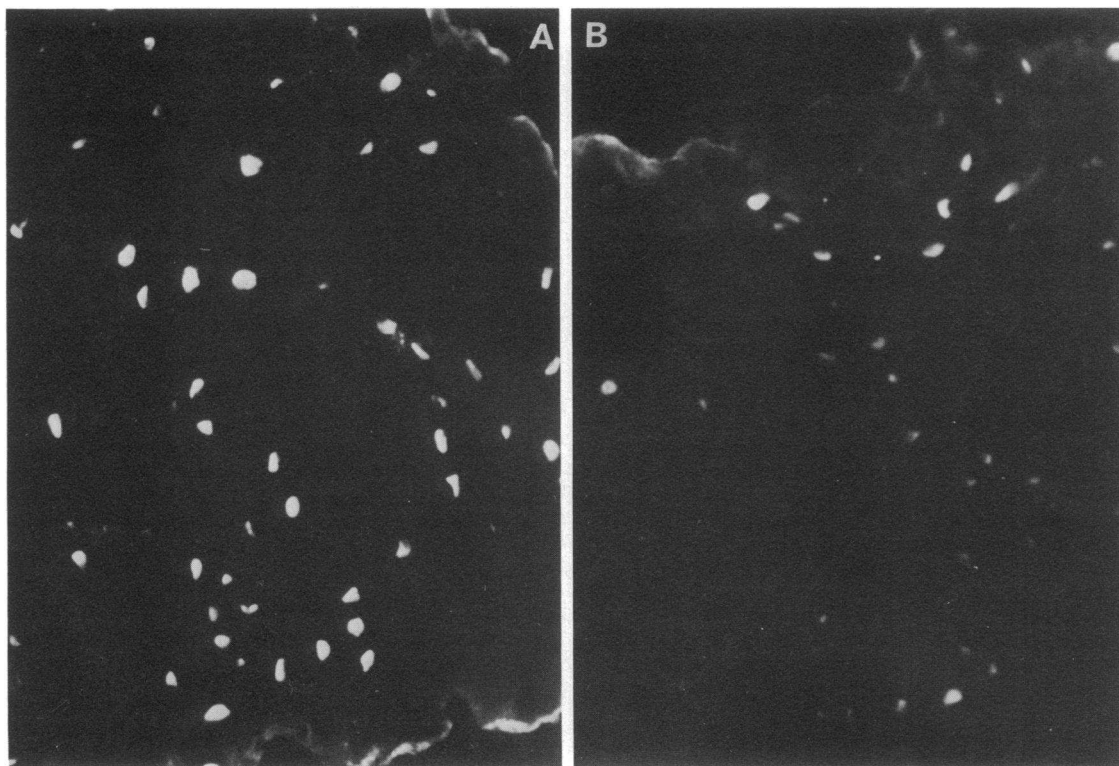


FIGURE 3 Immunohistochemical demonstration of lack of penetration of human alpha 1-antitrypsin into slices of rabbit articular cartilage. Details as in Fig. 2, except that whole human serum was incubated with the cartilage rather than enzyme, and rabbit anti-human alpha 1-antitrypsin antiserum was the primary stain (A). Normal rabbit serum was used as primary stain in the control (B). Original magnification  $\times 160$ .

steps, tissues were immediately frozen after removal from serum-containing solutions and the frozen sections prepared from these tissues were fixed before washing by one of three different fixation procedures (see Methods). The results shown in Fig. 3, obtained with sections of promptly frozen tissue fixed in cold acetone for 10 min before a 20-min immersion in buffered formalin, were typical. Under these conditions, the failure to detect proteinase inhibitor within the cartilage, after the latter's exposure to serum, was felt to be a true reflection of the inhibitor's inability to penetrate into such tissue.

*Parallel experiments with HRP-GARG as secondary stain.* Fig. 4A-C (bright field microscopy) show that essentially the same results were obtained when the foregoing experiments were repeated with HRP-GARG conjugate as the secondary stain in place of FITC-GARG. (Note that artifact chondrocyte staining was again visible).

## DISCUSSION

In the present work, two purified, human PMN neutral proteases have been tested for their effects on rabbit

articular cartilage in vitro. The first of these enzymes, PMN E, was originally described by one of the present authors (16, 28, 29), who also demonstrated its activity against synthetic E substrates (27, 30). Purification of the E (17, 21, 31-33) and its subsequent characterization (17, 21, 22, 33) have revealed that this PMN enzyme has a mol wt between 25,000 and 35,000 daltons and is a highly basic glycoprotein. The second enzyme, PMN chymotrypsin (CT), has been described by several laboratories (22, 23, 34), and this enzyme has also been purified recently (18, 24, 35). The mol wt of the principal CT isozyme lies between 23,000 and 28,000 daltons, and its amino acid composition has now been described with enzyme derived from both leukemic (35) and normal (18) cells. In an earlier report from this laboratory (15) it was suggested that these two PMN enzymes, E and CT, were the major proteases responsible for degradation of cartilage matrix proteoglycans by human PMN granule extracts (G) at neutral pH. This tentative conclusion was based on the observed solubilization of  $^{35}\text{S}$  from slices of rabbit articular cartilage by partly purified protein fractions isolated by preparative acrylamide gel electrophoresis



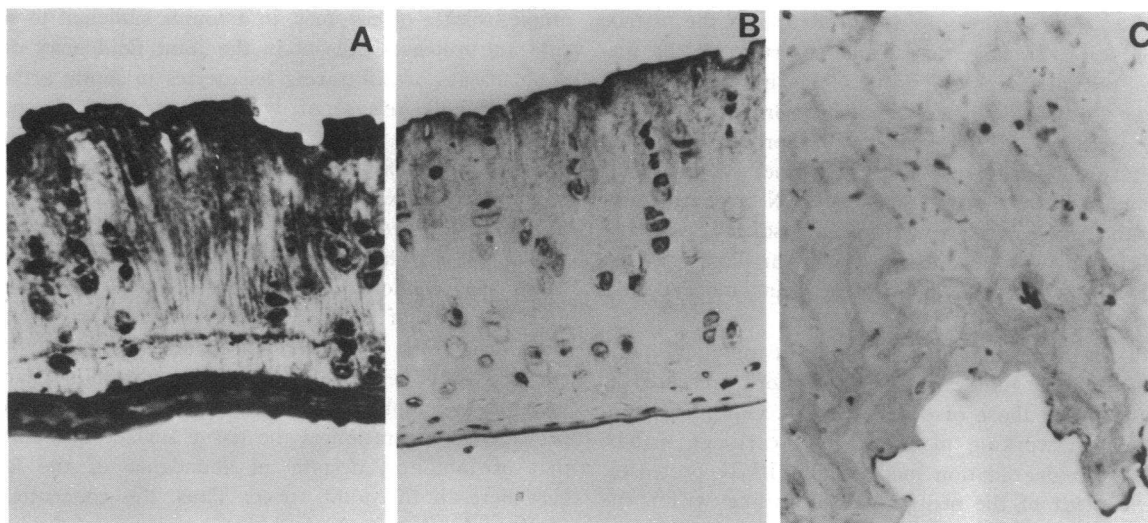


FIGURE 4 Immunohistochemical staining of rabbit articular cartilage slices exposed to human PMN enzymes or whole serum, as in the previous experiments. Details as in Figs. 2 and 3, except that the secondary stain was a goat antiserum (to rabbit immunoglobulins) conjugated with horseradish peroxidase rather than fluorescein. Peroxidase reaction product was developed with the diaminobenzidine reagent (see Methods). A and B, incubation with human PMN granule extract. C, incubation with human serum. Primary staining: A, rabbit anti-human PMN elastase antiserum; B, normal rabbit serum; C, rabbit antihuman alpha 1-antitrypsin antiserum. Original magnification  $\times 160$ .

of G. Only those acrylamide gel fractions containing E or CT were active in releasing the radiolabel (15).

In the present studies, this tentative conclusion has been further explored with highly purified PMN enzymes, and our results confirm the ability of both E and CT to release  $^{35}\text{SO}_4$  from articular cartilage slices at neutral pH. The initial lag in release of label (see Fig. 1) may have resulted from delayed diffusion of the enzymes into the cartilage slices, or it may reflect the time required for  $^{35}\text{SO}_4$ -containing digestion fragments to become small enough to allow their escape from the tissue into the incubation medium. Radiosulfate release can be inhibited by pretreatment of the enzymes with their specific chloromethyl ketone inactivators or with human alpha 1-antitrypsin, an endogenous proteinase inhibitor. However, several factors may be operating in inflamed joints, to reduce the effectiveness of the latter inhibitor under physiopathological conditions. These will be discussed in further detail below. In the present study, inhibition by antibody was also demonstrated in the case of E. Previous work by two of the present authors has shown that complexes of E and anti-E antibody retain hydrolytic activity against several low mol wt synthetic E substrates,<sup>9</sup> suggesting that most of the anti-elastase antibodies are not directed against the enzyme's active site. The inactivity of the complexes against macromolecular proteoglycans within cartilage suggests that steric factors probably prevented penetration of enzyme-

antibody complex into the tissue or prevented the interaction of the macromolecular tissue substrates with the active center of the enzyme.

In the studies reported here, release of radiosulfate from cartilage slices is taken as an index of solubilization of chondroitin sulfate- and/or keratan sulfate-containing peptides from degraded cartilage matrix proteoglycan. Other studies (9, 10) with labeled cartilage from different tissue sources have demonstrated that  $^{35}\text{SO}_4$  released by PMN proteases is almost entirely precipitable by cetyl trimethyl ammonium bromide, an indication that the released isotope is still bound to mucopolysaccharides. Data presented in the accompanying paper (14) will show that the purified PMN enzymes used here are capable of degrading isolated cartilage proteoglycans, to which the vast bulk of the  $^{35}\text{SO}_4$  incorporated into hyaline cartilage fixes (12, 13). Moreover, it is also evident from the accompanying paper (14) that contaminating endoglycanohydrolases were not responsible for isotope release by the purified PMN proteases, since our preparations of E and CT and even G were incapable of degrading purified chondroitin sulfate or hyaluronic acid under the conditions used in the incubations with cartilage.

On the other hand, our data do not exclude the possibility that E and CT produce some alteration in cartilage matrix structure that in turn allows enzymes intrinsic to cartilage (extracellular or intracellular en-



zymes) to then participate in degradation of the matrix proteoglycans. If true, this would not rule out the important role of the PMN proteases as initiators of the pathway leading to damage, whether or not other enzymes participated in the final injury process. The important role of PMN proteases is further supported by the demonstration that the purified PMN enzymes cause rapid and extensive degradation of isolated cartilage proteoglycan (14), whereas intrinsic cartilage enzymes appear to degrade isolated proteoglycans more slowly and less extensively.<sup>3</sup>

With the foregoing considerations in mind, it might now be useful to integrate the observations presented in this paper with those of other workers. Our purpose is to develop a working model of joint cartilage matrix proteoglycan degradation mediated by PMN proteases, in the context of the neutral pH prevailing within inflamed joints (6). The first step in the process would occur when bacteria, viruses, microcrystalline deposits (e.g., monosodium urate), or antigen-antibody complexes appear in the synovial spaces. These materials may be present as a particulate suspension throughout the synovial fluid, or as an adsorbed layer on articular surfaces, or both. The second step involves the liberation of intrinsic, diffusible factors (e.g., by certain bacteria) or the activation of the complement system (e.g., by antigen-antibody complexes) to form a concentration gradient of PMN chemoattractants. This, in turn, leads to a chemotactic response and the emigration of large numbers of PMN into the joint space. In step three of the model, PMN leukocytes entering the joint fluids proceed to engulf the suspended particulates or attempt to engulf particulates adsorbed onto cartilage surfaces and, in so doing, release quantities of their lysosomal enzymes into the synovial fluid or directly onto the articular cartilage surfaces. These initial steps in the model's sequence (chemotaxis, phagocytosis, lysosomal enzyme secretion) have already been suggested by many other workers and are based on their detailed experimental observations of PMN behavior in *in vitro* analogues of joint inflammation (for recent reviews, see refs. 5 and 36).

Once lysosomal enzymes have been released from leukocytes in the joint space, endogenous antiproteases present in synovial fluid (25, 26, 37, 38) may act to protect susceptible substrate molecules from rapid digestion by the released enzymes. It is worth bearing in mind, however, that under certain conditions in the joint, the formation of inhibitory complexes between leukocyte proteases and synovial antiproteases may be prevented and the enzymes may remain active. For ex-

ample, release of enzymes, in amounts sufficient to saturate antiproteases present in the joint fluid, may occur from masses of infiltrating leukocytes in septic arthritis or after bulk release of lysosomal contents from injured and dying neutrophils in gout. The concentration of free antiproteases may also become significantly lowered by complexing with bacterial proteases in septic joints, as has been suggested in the case of lung infection (39). In rheumatoid arthritis, a different mechanism may interfere with efficient removal of proteases that leak out of neutrophils. If enzymes are released from cells while the latter are in direct contact with a connective tissue target (as from neutrophils attached to immune complexes adsorbed onto cartilage surfaces), protease concentration at the tissue surface may exceed that of inhibitor, despite an abundance of the latter elsewhere in the joint space. Thus, the concentration of free inhibitors in the bulk phase of synovial fluid may not always accurately reflect their local concentration in the immediate vicinity of the target tissue. If one adds to this the likelihood that penetration of a major antiprotease (alpha 1-antitrypsin) into cartilage matrix is much less efficient than penetration of leukocyte neutral proteases, then the possibility of matrix damage by PMN proteases in inflamed joints would be further increased. The inability of alpha 1-antitrypsin to penetrate into intact cartilage was demonstrated immunohistochemically in the present work. This correlates well with the observation that cartilage pretreated with alpha 1-antitrypsin, and then secondarily exposed to PMN protease in the absence of the inhibitor, remains fully susceptible to enzyme-mediated radiolabeled release. Under identical conditions, lower molecular weight inhibitors protect cartilage against PMN protease.<sup>4</sup>

Finally, several unresolved questions that affect the foregoing model should be briefly mentioned. First, the penetration of alpha 1-antitrypsin into cartilage matrix may be enhanced if the tissue has already been altered by exposure to PMN proteases. Of course, inhibitor penetration dependent on prior injury to tissue would not be likely to combat the injury process effectively. (We attempted to investigate this possibility by indirect immunofluorescence with cartilage exposed to G before incubation with serum. However, indirect immunofluorescence proved unsatisfactory because our rabbit antiserum to human alpha 1-antitrypsin cross-reacted with G. This observation was not surprising, since proteinase inhibitor in the circulation could be partially complexed with leukocyte proteases, and an antiserum raised against the inhibitor could contain antibodies to the attached enzymes as well.) Second, an undefined

<sup>3</sup> Sapolsky, A., H. Keiser, J. F. Woessner, and D. Howell. Submitted for publication.

<sup>4</sup> Feinstein, G., C. J. Malemud, and A. Janoff. Manuscript submitted for publication.

proteinase inhibitory activity has recently been described in extracts of animal cartilage (40). Whether such endogenous cartilage inhibitors exist in man and whether they affect PMN neutral proteases seem important subjects for future study.

The model of cartilage matrix damage by PMN enzymes presented above will be extended to the molecular level in the following paper (14). It is to be expected that this working model of cartilage proteoglycan attack by PMN neutral proteases in joint disease will require significant revision over the course of time. In the meanwhile, it may serve to stimulate further studies aimed at devising effective methods to impede this degradative process. One such study might be aimed at the development of inhibitors of PMN neutral proteases capable of effectively penetrating or even concentrating in joint cartilage.

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