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Evidence for polymorphonuclear-leucocyte-derived proteinases in arthritic cartilage

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1. An enzyme that degrades proteoglycan at neutral pH was extracted with 4M-guanidine hydrochloride from the articular cartilage of rabbits with antigen-induced arthritis. 2. The enzyme had an apparent molecular weight on Ultrogel AcA 54 of about 8000 and was optimally active at pH 7.5 in Tris/HCl buffer containing 0.2M-NaCl. The partially purified preparation was totally inhibited by 0.01mM-*N*-acetyldialanylprolylvalylchloromethane, severely inhibited by 2mM-phenylmethanesulphonyl fluoride and soya-bean trypsin inhibitor (200μg/ml) and slightly inhibited by 10mM-EDTA. Marked inhibition was also obtained with a cytosolic fraction prepared from rabbit polymorphonuclear leucocytes. 3. All properties of the enzyme were virtually identical with those of an 'elastase-like' proteinase that was isolated from rabbit polymorphonuclear-leucocyte granules. 4. The results are consistent with the idea that cartilage proteoglycan degradation in acute joint inflammation is due at least partly to the diffusion into the cartilage of proteinases derived from synovial-fluid polymorphonuclear leucocytes.

Acute antigen-induced arthritis in rabbits exhibits many features characteristic of the early phase of human rheumatoid arthritis (Dumonde & Glynn, 1962). Induction of the experimental arthritis is followed by the influx to the synovial fluid of large numbers of polymorphonuclear leucocytes and the depletion of up to 40% of the articular-cartilage proteoglycan (Lowther *et al.*, 1978). This loss is accompanied by a very marked inhibition of chondrocyte proteoglycan synthesis and the complete degeneration of superficial layer cells in weight-bearing areas. It appears that proteoglycan loss results from the combined effects of accelerated degradation and inhibited synthesis, and it is possible that both these changes result from elevated activities of proteinases in the cartilage matrix (Sandy *et al.*, 1980). However, the presence of such proteinases has not been directly demonstrated. Further, although it is clear that such enzymes may be derived from polymorphonuclear leucocytes, synovial fibroblasts, macrophages or chondrocytes, the quantitative importance of enzymes from these sources in acute and chronic inflammation remains unknown (Barrett, 1975; Dingle, 1979).

The work described here directly demonstrates elevated neutral-proteinase activities in cartilage during acute experimental arthritis, and indicates that the activity is derived from the synovial-fluid polymorphonuclear leucocytes.

Abbreviations used: NAc-Ala$_2$-Pro-Val-CH_2Cl, *N*-acetyldialanylprolylvalylchloromethane; Hepes, 4-(2-hydroxyethyl-1-piperazine-ethanesulphonic acid.

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Experimental

Materials

Cytochalasin B (from *Helminthosporium dematioideum*), cyclic GMP (sodium salt), benzamidine hydrochloride, phenylmethanesulphonyl fluoride, 6-aminohexanoic acid, soya-bean trypsin inhibitor (type 1-S), 4-aminophenylmercuric acetate, 4-chloromercuribenzoic acid, trypsin (EC 3.4.21.4, bovine pancreas, type III) and elastase (EC 3.4.21.11, porcine pancreas, type I) were from Sigma. Cyanogum-41, *NNN'N'*-tetramethylethylenediamine and Brij-35 were from BDH. Rabbit γ-globulin (B grade) and chymotrypsin (EC 3.4.21.1, bovine pancreas, A grade) were from Calbiochem. Collagenase (type I from *Clostridium histolyticum*) was from Worthington Biochemical Corp. and Ultrogel AcA 54 was from LKB Producter. Human serum albumin (Cohn fraction V) was from Commonwealth Serum Laboratories,

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Melbourne, Australia. NAc-Ala_2-Pro-Val-CH_2Cl was kindly given by Professor J. Powers, School of Chemistry, Georgia Institute of Technology, Atlanta, GA 30332, U.S.A. NCS tissue solubilizer was from Amersham Corp., Arlington Heights, IL 60005, U.S.A. Trasylol (Aprotinin) was from Bayer Pharmaceutical Co., Botany, N.S.W. 2019, Australia. Sources of all other materials were given previously (Sandy *et al.*, 1978, Sandy, 1979).

Methods

Preparation of ^{35}S-labelled cartilage proteoglycan. Bovine articular cartilage from the metacarpal–phalangeal joints of adult steers was obtained from a local abattoir and used within 1h of death of the animal. Cartilage (about 30g) was sliced finely, washed and added to 150ml of a medium containing: 120mM-NaCl, 5.4mM-KCl, 0.98mM-$MgCl_2$, 0.96mM-NaH_2PO_4, 5.6mM-glucose, 1.8mM-$CaCl_2$, 1mM-glutamine, 5mM-glutamate, 5mM-sodium fumarate, 5mM-sodium pyruvate and 25mM-Hepes buffer, pH7.4. After 30min incubation at 37°C under O_2/CO_2 (19:1), 6mCi of carrier-free $Na_2{}^{35}SO_4$ was added and incubation was continued for 6h. The radioactive medium was removed, the cartilage was washed with five 100ml portions of phosphate-buffered saline (Dulbecco & Vogt, 1954) and then extracted for 48h at 4°C in 200ml of 4M-guanidine hydrochloride [containing 50mM-Tris/HCl buffer, pH7.5, 10mM-sodium EDTA, 5mM-benzamidine hydrochloride, 0.1mM-phenylmethanesulphonyl fluoride, 100mM-5-aminohexanoic acid, soya-bean trypsin inhibitor (1μg/ml) and 1mM-iodoacetic acid]. The cartilage residue was removed by filtration on six layers of gauze and the extract was adjusted to 1.60g/ml with solid CsCl before centrifugation in an MSE-75 centrifuge at 35000rev./min ($r_{av.}$ = 7.72cm) for 44h at 12°C in an 8 × 50ml angle rotor. The contents of the bottom two-fifths of each tube were combined and dialysed exhaustively at 4°C against water, 3M-NaCl and finally water, for 48h in each case. The dialysis residue was freeze-dried and the product, generally about 0.5g, was stored at 20–25°C in a desiccator. [^{35}S]Proteoglycan prepared in this manner contained about 20% (w/w) hexuronic acid and the specific radioactivity was about 10^6d.p.m./mg of proteoglycan.

Preparation of polyacrylamide discs containing [^{35}S]proteoglycan. [^{35}S]Proteoglycan (20mg) was dissolved by gentle mixing in 1.5ml of 0.1M-Tris/HCl buffer, pH7.4, to which was added 0.5ml of a 20% (w/v) solution of Cyanogum-41 and 20μl of a 10% (v/v) solution of *NNN'N'*-tetramethylethylenediamine. The mixture was deaerated under reduced pressure, 20μl of a freshly prepared 10% (w/v) solution of ammonium persulphate was added and the thoroughly mixed solution was pipetted into glass tubing (5mm internal diameter) sealed at one end with Parafilm. Ater 1h at 20–25°C the set gel was removed from the tubing, partially frozen with solid CO_2 on the stage of a Mickle gel slicer (Mickle Laboratories, Gomshall, Surrey, U.K.) and cut into discs of 0.8mm thickness. The discs (about 100) were washed in 0.15M-NaCl and stored at 4°C in 0.15M-NaCl (containing 0.64mg of streptomycin sulphate and 0.27mg of sodium benzylpenicillin/ml). Each disc generally contained 110–150μg (up to 180000d.p.m.) of [^{35}S]proteoglycan. Discs could be stored at 4°C for up to 4 weeks without significant loss of substrate content or reactivity.

Assay of proteoglycan-degrading enzymes. Assays were done in screw-top glass or plastic vials (1.2cm × 5cm) and contained 0.05mmol of Tris/HCl buffer, pH7.4, 5μmol of $CaCl_2$ 5μmol of $MgCl_2$, 0.1mmol of NaCl, enzyme extract and one [^{35}S]proteoglycan polyacrylamide disc in a total volume of 0.5ml. Substrate discs were washed in water and blotted dry immediately before addition to the assay. Incubation was at 37°C in a shaking water bath set at 30–40 cycles/min. After incubation (6–24h) the complete supernatant was removed and retained, the substrate disc was washed with 0.5ml of distilled water and the washing was added to the supernatant for radioactivity measurement (degradation product). The disc was washed a second time with 0.5ml of water, the washing was discarded and the disc was treated with 0.2ml of NCS tissue solubilizer for 2h at 60°C followed by the addition of 0.8ml of water for radioactivity measurement (undegraded substrate). The radioactivity content of the degradation product (1.0ml) and the undegraded substrate (1.0ml) in each assay was determined by the addition of 4ml of toluene/Triton X-114 [1:1, v/v; containing 8g of 2,5-diphenyloxazole (PPO)/litre] and counting for radioactivity in a Philips automatic liquid-scintillation analyser.

Proteoglycan-degrading-enzyme activity units were calculated from the radioactivity content of the degradation product and the specific radioactivity of the [^{35}S]proteoglycan substrate. One unit of activity is defined as that amount that results in the release of 1μg (about 1000d.p.m.) of [^{35}S]proteoglycan/min under the conditions of the assay. All activities were corrected for non-enzymic release of [^{35}S]proteoglycan, which was generally 3–7% of the total disc substrate over an 18h assay period.

In each assay the total [^{35}S]proteoglycan radioactivity in the degradation product plus the undegraded substrate was calculated as a check on the uniformity of the size and substrate content of the discs. In a typical assay (39 discs) the total radioactivity per assay was 154296 ± 14052d.p.m. (mean ± S.D.).

Induction of the arthritis. The methods for

immunization, joint injection and general handling of rabbits were described previously (Lowther & Gillard, 1976; Lowther *et al.*, 1978). The antigen used in all experiments was human serum albumin, and all samples were obtained from rabbits killed 72h after joint injection.

Collection of synovial fluid and preparation of cells. The hind legs were removed immediately after killing the rabbits and the joint contents were recovered by aspiration with a syringe after two injections of 1.0ml of 0.15M-NaCl. The joint was opened and synovial fluid was recovered from the surface in another 3.0ml of 0.15M-NaCl. The synovial fluid and washings (about 5ml/joint) were filtered through six layers of gauze and the cell suspension was centrifuged at 200*g* for 10min at 4°C. After hypo-osmotic lysis of erythrocytes for 30s, the NaCl concentration was adjusted to 0.15M and the intact cells were recovered by centrifugation at 200*g* for 10min at 4°C. For differential cell counts, smears were prepared from this pellet followed by fixation for 30 min in methanol and staining with 5% (v/v) Giemsa in sodium citrate buffer, pH5.75, for 30min at 4°C.

Preparation of glycogen-induced peritoneal exudates. This method is a modification of that previously described (Cohn & Hirsch, 1960). Adult laboratory rabbits (weighing more than 2.5kg) were injected intraperitoneally with 150ml of 0.1% (w/v) glycogen in 0.15M-NaCl. After 18h, rabbits were anaesthetized with Nembutal and the peritoneal exudate was collected by gravity drainage. Normally 100ml of exudate containing about 5×10^8 cells was obtained per rabbit. The cell suspension was filtered through six layers of gauze, and contaminating erythrocytes were removed by hypo-osmotic lysis. A differential cell count was performed on smear preparations prepared as described for synovial-fluid cells. The average viability was 97% and polymorphonuclear leucocytes accounted for at least 94% of the total cells present.

Extraction of proteinase activity from synovial-fluid cells. This method is an adaptation of those described by Cohn & Hirsch (1960) and Higuchi *et al.* (1975). Cells were washed by centrifugation in 0.15M-NaCl, resuspended in 3ml of 0.01M-citric acid and stirred gently for 16h at 4°C. The suspension was centrifuged at 8200*g* for 15min at 4°C and the clear supernatant was adjusted to pH7.4 with Tris base and stored at −20°C.

Subcellular fractionation of polymorphonuclear leucocytes and extraction of granules. This method is a modification of that previously described by Cohn & Hirsch (1960). The procedure was carried out between 0 and 4°C. The cells (about 5×10^7) were washed once in 0.34M-sucrose, resuspended in 2ml of 0.34M-sucrose and sonicated (3s burst, 70% duty cycle, setting no. 3) with a Branson sonifier.

The sonicated material was centrifuged at 500*g* for 15min and the supernatant was retained. The pellet was suspended in 2ml of 0.34M-sucrose, sonicated and centrifuged as above and the combined supernatants were centrifuged at 12000*g* for 15min. The supernatant (cytosol fraction) was stored at −20°C and the pellet (granule fraction) was washed once by suspension in 5ml of 0.34M-sucrose and centrifugation at 12000*g* for 15min. Granules were extracted by stirring in 2ml of 0.01M-citric acid for 16h at 4°C. The extract was centrifuged at 12000*g* for 15min and the supernatant (granule extract) was adjusted to pH7.4 with Tris base and stored at −20°C.

Preparation of heat-aggregated rabbit γ-globulin. Rabbit γ-globulin (156mg) was suspended in 6ml of Hanks salt solution (Hanks & Wallace, 1949), heated at 60°C for 40min and stored at 4°C for 16h. The aggregated protein was recovered by centrifugation at 30000*g* for 30min and the precipitate was resuspended by gentle homogenization in cold Hanks salt solution to a final protein concentration of 4mg/ml.

Secretion of enzymes from synovial-fluid-cell suspensions. This method is an adaptation of those described by Weissmann *et al.* (1975) and Ignarro (1974*a*) for the stimulation of granule-enzyme release from human polymorphonuclear leucocytes. Cells (about 5×10^7) prepared from synovial fluid as described above were suspended in 3ml of Hanks salt solution containing 10mM-$CaCl_2$. To this was added 3μl of cytochalasin B (5mg/ml solution in dimethyl sulphoxide) and 3μl of cyclic GMP (0.1mM solution in Hanks salt solution). This mixture was incubated in a shaking water bath at 40 cycles/min at 37°C for 10min, 1.5ml of freshly prepared heat-aggregated rabbit γ-globulin solution (4mg/ml in Hanks salt solution) was added, and incubation was continued for 30min. The cells were removed by centrifugation at 500*g* for 10min at 4°C and the supernatant was clarified by centrifugation at 12000*g* for 20min and stored at −20°C. Samples prepared in this way were free of lactate dehydrogenase activity, consistent with the discrete secretion of granule contents without significant cell lysis.

Extraction of proteinase activity from articular cartilage. Cartilage was dissected from articular surfaces, taking care to exclude surrounding soft tissue and subchondral bone. The finely sliced tissue (50–200mg wet wt.) was washed four times in 10ml of cold 0.05M-Tris/HCl, pH7.4, and extracted by gentle stirring for 24h at 4°C in 3ml of 4M-guanidine hydrochloride/0.1M-Tris/HCl, pH7.4. The cartilage residue was removed by filtration on cottonwool, the filtrate was dialysed for 24h at 4°C against 1M-NaCl/50mM-Tris/HCl(pH7.4)/10mM-$CaCl_2$/10mM-$MgCl_2$ and stored at −20°C.

Incubations with proteinase inhibitors. Enzyme (50–100 μl) was preincubated for 1 h at 22–25°C in a total volume of 0.5 ml with all assay components and the appropriate concentration of proteinase inhibitor. The assay was started by addition of the substrate disc. Stock solutions of inhibitors were 0.2 M-phenylmethanesulphonyl fluoride (in propan-1-ol), 1 mg of soya-bean trypsin inhibitor/ml, 0.1 M-sodium EDTA, pH 7.5, 10 mM-4-chloromercuribenzoate (in 0.1 M-glycylglycine buffer, pH 8) and 1 mM-NAc-Ala_2-Pro-Val-CH_2Cl (in methanol). Control incubations with appropriate volumes of inhibitor solvents produced no significant inhibition of activity.

Column chromatography. Samples (about 2 ml) were fractionated on a column (1.6 cm × 70 cm) of Ultrogel AcA 54 eluted at about 12 ml/h with 1 M-NaCl/50 mM-Tris/HCl(pH 7.4)/10 mM-$CaCl_2$/10 mM-$MgCl_2$/0.05% Brij-35/0.02% NaN_3. The column was calibrated by elution of a mixture of ^{35}S-labelled proteoglycan (V_0), bovine serum albumin (mol.wt. 68000), ovalbumin (mol.wt. 45000), soya-bean trypsin inhibitor (mol.wt. 22000), Trasylol (mol.wt. 6500) and inorganic [^{35}S]sulphate (V_t).

Results and discussion

Assay of proteoglycan-degrading enzymes

The method used in this work is an adaptation of those described by Barrett (1966) and Dingle *et al.* (1977). Both the specific radioactivity of the substrate and the total substrate used per assay are similar to the polyacrylamide bead assay (Dingle *et al.*, 1977); however, the disc composition [4.75% (w/v) acrylamide with 0.25% (w/v) methylenebisacrylamide] is about one-third the polyacrylamide concentration used in the beads. It was found in assays with trypsin that the lower polyacrylamide concentration improved the assay sensitivity about 3-fold with no marked change in the degree of retention of undegraded substrate.

The effect of both incubation time and enzyme concentration on substrate degradation was determined with trypsin. With 0.05 μg of trypsin per assay the rate of substrate degradation, about 4 μg of [^{35}S]proteoglycan/h, was essentially linear with time for up to 18 h of incubation, resulting in release of about 65% of the substrate; this rate was, however, markedly decreased on further incubation. During an 18 h incubation the amount of substrate degraded was linearly related to the enzyme concentration between 0.01 μg and 0.05 μg of trypsin, that is, between 10 and 65% release of substrate. Higher concentrations of enzyme produced less than the expected degradation, with 0.10 μg and 0.50 μg of trypsin resulting in only 73 and 87% release respectively. In the quantitative work described below, the incubation time was generally 18 h and the amount of enzyme was adjusted to obtain results in the linear region of the assay, that is between 10 and 65% release of product. The reproducibility of the assay was assessed by including 0.05 μg of trypsin as a positive control in 21 separate assays carried out over a period of two months. For an 18 h incubation period the percentage release was 65.6 ± 3.0 (mean ± S.D.), showing that the assay provides a high degree of reproducibility between different assays and different disc preparations.

The release of [^{35}S]proteoglycan from substrate discs did not exceed 6% of the total in 20 h blank assays with 0.1 M-sodium acetate buffer, pH 4–5.5, 0.1 M-sodium cacodylate buffer, pH 5–7.5, 0.1 M-sodium phosphate buffer, pH 6–7.5 and 0.1 M-Tris/HCl buffer, pH 7–9. This form of [^{35}S]proteoglycan substrate is therefore useful for proteinase assays in the range from pH 4–9. The release of substrate from discs in 20 h blank incubations with 0.1 M-Tris/HCl, pH 7.4, was also not significantly affected by the inclusion of NaCl in the concentration range from 0.05 M to 1 M. This method is therefore applicable to the assay of neutral proteinases in the presence of the high salt concentrations often used in fractionation procedures with these enzymes.

Demonstration of proteoglycan-degrading activity in extracts of arthritic cartilage

Previous radioisotope experiments *in vitro* with tissue from rabbits with antigen-induced arthritis had shown a marked inhibition of chondrocyte proteoglycan synthesis in arthritic cartilage (Sandy *et al.*, 1980). To investigate the structure of the newly synthesized proteoglycans, cartilage was extracted with guanidine hydrochloride and the extracts were dialysed against 0.01 M-sodium acetate buffer, pH 6.8, and fractionated on Sepharose CL-2B. It was found that, relative to controls, the proteoglycan in extracts of arthritic cartilage was extensively degraded, resulting in almost total loss of hyaluronic acid-binding properties. These results suggested that such extracts may contain a proteoglycan-degrading enzyme active at pH 6.8 during sample preparation. Indeed, when guanidine extracts of arthritic cartilage were dialysed (see under 'Methods') and assayed at a range of concentrations for proteoglycan-degrading activity, substrate degradation was found to be essentially linear with volume of extract assayed up to about 0.04 enzyme unit (Fig. 1). The yield of activity was increased only slightly by increasing the period of extraction from 24 to 48 h, and the extracted activity was stable to storage at −20°C for at least 5 weeks. No activity could be demonstrated in the washings of the fresh cartilage (see under 'Methods'). However, about 10–20% of the activity could be recovered by extraction in

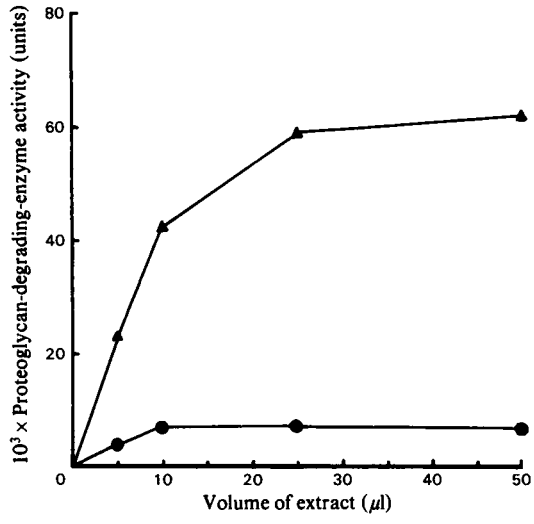

Fig. 1. *The assay of proteoglycan-degrading activity in extracts of arthritic cartilage*

Extracts prepared from arthritic cartilage (see the text for details) were assayed at a range of concentrations for proteoglycan-degrading activity. The guanidine hydrochloride extract was assayed after dialysis against 1 M-NaCl/50 mM-Tris/HCl(pH 7.4)/10 mM-$CaCl_2$/10 mM-$MgCl_2$ (▲); the 0.2 M-NaCl extract was assayed without further treatment (●).

Table 1. *Distribution of proteoglycan-degrading activity in cartilage from different anatomical sites*

Cartilage from different regions was extracted in 4 M-guanidine hydrochloride/0.1 M-Tris/HCl, pH 7.4 and portions (5, 10, 25 and 50 μl) of the dialysed extracts were assayed for proteoglycan-degrading activity.

		Proteoglycan-degrading activity (units/100 mg wet wt.)		
Rabbit	Anatomical site ...	Femoral condyles	Tibial plateaux	Patellar groove
1		10.84	7.42	2.80
2		14.22	6.08	9.10
3		7.12	7.64	5.68
4		8.74	7.32	7.74

0.2 M-NaCl rather than 4 M-guanidine hydrochloride (Fig. 1).

There was no marked difference in the amount of enzyme present in different regions of cartilage (Table 1). Further, the meniscal cartilages from two arthritic joints were found to contain 7.44 units/100 mg wet weight, an activity similar to that found in articular cartilage. The amount of enzyme in the articular cartilage of rabbits with carageenin-induced arthritis of 24 h duration (Lowther & Gillard, 1976) was similar to that described above.

Extracts prepared from normal control cartilages showed no activity when assayed alone or in the presence of 1 mM-4-aminophenylmercuric acetate, to detect latent proteinases. Further, no activity could be detected in fractions from chromatography of normal extracts on Ultrogel AcA 54 (see below).

Characterization of activity in extracts of arthritic cartilage

When enzyme from arthritic cartilage was fractionated on Ultrogel AcA 54 (Fig. 2), activity was recovered over a rather wide elution range between 90 and 140 g of eluant, with a peak of activity corresponding to an apparent molecular weight of 8300. Assay of these fractions in the presence of a range of proteinase inhibitors showed that they all contained serine-dependent proteinase activity; thus phenylmethanesulphonyl fluoride was severely inhibitory (70–90% inhibition), as was soya-bean trypsin inhibitor (60–80% inhibition). As much as 50% inhibition was also obtained with EDTA and 10–20% inhibition with 4-chloromercuribenzoate. The activity of all fractions was also totally inhibited by 0.01 mM-NAc-Ala_2-Pro-Val-CH_2Cl, a potent inhibitor of human leucocyte elastase (Powers *et al.*, 1977). The isolated enzyme was also sensitive to inhibition by a soluble fraction isolated from rabbit polymorphonuclear leucocytes (see under 'Methods'), 50% inhibition being obtained with 80 μg of cytosolic protein.

The enzyme isolated from cartilage extracts showed a critical dependence on NaCl in the assay, being optimally active at a concentration of 0.2 M (Fig. 3). A study of the effect of pH and buffer composition on activity (Fig. 4) showed the enzyme to be optimally active in Tris buffer at pH 7.5. Relatively high activity was also observed in cacodylate buffer at pH 6.5, however, the preparation was inactive in acetate buffer below pH 5, consistent with the absence of significant amounts of cathepsin D or other proteinases.

Investigation of the source of the enzyme in cartilage extracts

The enzyme isolated from arthritic cartilage may have been derived from the chondrocytes, or from a cell-type external to the cartilage by diffusion into the articular surface from the joint space. Possible external sources would include synovial fibroblasts, macrophages and polymorphonuclear leucocytes.

To investigate this, synovial fluid was collected from arthritic joints and the cells were separated by centrifugation (see under 'Methods'). The cell-free

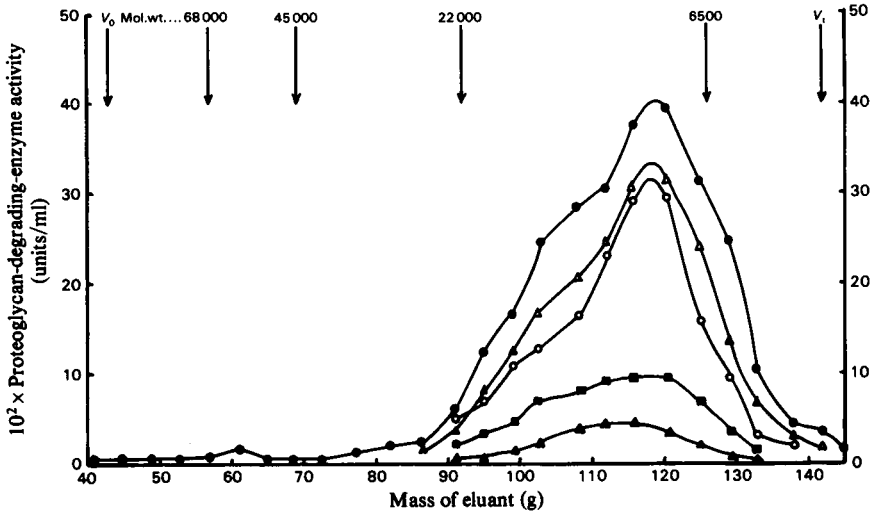

Fig. 2. *Chromatography on Ultrogel AcA 54 of proteinases extracted from arthritic cartilage and the effect of proteinase inhibitors on the activity*

A guanidine hydrochloride extract of arthritic cartilage (1.5 ml, 15.0 units of enzyme) was fractionated on a column (1.6 cm × 70 cm) of Ultrogel AcA 54 and eluted with 1 M-NaCl/50 mM-Tris/HCl(pH 7.4)/10 mM-$CaCl_2$/10 mM-$MgCl_2$/0.05% Brij-35/0.02% NaN_3. Fractions (about 4 g) were collected at a flow rate of 12 ml/h and portions (100 μl) were assayed for activity without further treatment (●), and in the presence of 1 mM-4-chloromercuribenzoate (△), 10 mM-EDTA (○), 200 μg of soya-bean trypsin inhibitor/ml (■) and 2 mM-phenylmethanesulphonyl fluoride (▲). The recovery of activity from the column was 71%.

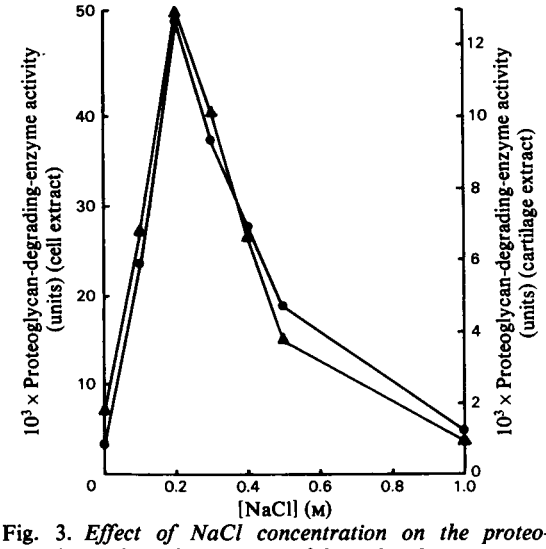

Fig. 3. *Effect of NaCl concentration on the proteoglycan-degrading activity of the isolated enzymes*

Enzyme recovered from chromatography on Ultrogel AcA 54 between 115 and 125 g of eluant (Fig. 2 and Fig. 5) was dialysed for 16 h at 4°C against 0.1 M-Tris/HCl(pH 7.4)/10 mM-$CaCl_2$/10 mM-$MgCl_2$ and the effect of NaCl concentration on the proteoglycan-degrading activity was determined. ●, Enzyme (100 μl/assay) from cartilage extract; ▲, enzyme (50 μl/assay) from synovial-fluid-cell extract.

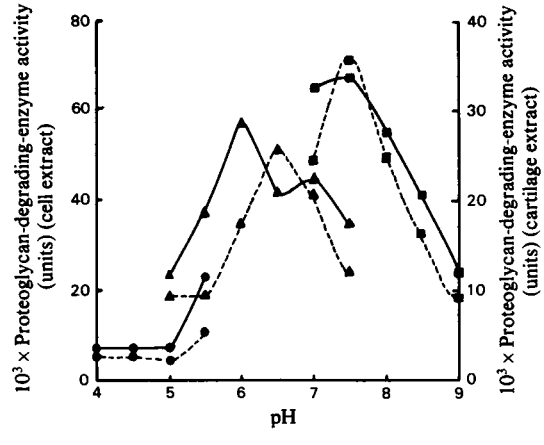

Fig. 4. *Effect on pH on the proteoglycan-degrading activity of the isolated enzymes*

Enzyme recovered from chromatography on Ultrogel AcA 54, between 115 and 125 g of eluant (Fig. 2 and Fig. 5), was dialysed for 16 h at 4°C against 0.2 M-NaCl/10 mM-$CaCl_2$/10 mM-$MgCl_2$ and assayed for proteoglycan-degrading activity at a range of pH values in 0.1 M-acetate buffer (●), 0.1 M-cacodylate buffer (▲) and 0.1 M-Tris buffer (■). ——, Cell extract (50 μl per assay); – – – –, cartilage extract (100 μl per assay).

synovial fluid contained no demonstrable proteoglycan-degrading activity at pH 7.4. Further, there was no evidence for latent proteinase in these fluids; thus no activity was generated by dialysis against 3 M-NaSCN or 1 M-NaCl, nor by trypsin treatment (0.25 μg/ml for various periods up to 2 h at 37°C) followed by addition of soya-bean trypsin inhibitor (1 μg/ml). Indeed 5 μl of synovial fluid completely inhibited 0.05 unit of enzyme extracted from synovial-fluid cells (see below), indicating that a large excess of serum inhibitors, presumably α_2-macroglobulin and α_1-antitrypsin, was present in these fluids.

The pellet isolated from the synovial fluid contained between 10^7 and 5×10^7 cells, and was composed of about 50% polymorphonuclear leucocytes, the remainder being monocytes and lymphocytes (Lowther *et al.*, 1978). To examine proteoglycan-degrading activity in these cells, preparations were treated either by (*a*) suspension in 0.05 M-phosphate buffer, pH 7.5, followed by ten freeze–thaw cycles (Ignarro *et al.*, 1973) or (*b*) homogenization in 0.05 M-phosphate buffer, pH 7.5, containing 0.1% Triton X-100 (Weissmann *et al.*, 1969) or (*c*) extraction in 10 mM-citric acid for 16 h at 4°C (Higuchi *et al.*, 1975). Although some activity was obtained by all methods, it was found that citric acid extraction consistently gave the highest yields (about 0.10 unit/20 μg of soluble protein). The total proteoglycan-degrading activity in these extracts varied between 5.0 and 27.0 units/10^7 cells.

The relationship between this activity and that isolated from the cartilage (Fig. 2) was investigated by fractionation on Ultrogel AcA 54 (Fig. 5). The activity was recovered in a symmetrical peak between 100 and 135 g of eluant corresponding to an apparent mol. wt. of 7800. This enzyme was therefore very similar in chromatographic behaviour to the major active species in the cartilage extract (Fig. 2).

The effect of proteinase inhibitors on this activity (Fig. 5) was also very similar to that observed with the enzyme extracted from arthritic cartilage. Thus severe inhibition was observed with phenylmethanesulphonyl fluoride and soya-bean trypsin inhibitor, marked inhibition with EDTA and only slight inhibition with 4-chloromercuribenzoate. Further, total inhibition of all fractions was obtained with 0.01 mM-NAc-Ala$_2$-Pro-Val-CH_2Cl, and 50% inhibition was obtained with 30 μg of cytosolic protein from rabbit polymorphonuclear leucocytes.

The enzyme isolated from synovial cells showed a dependence on NaCl concentration for activity that was virtually identical with the cartilage enzyme

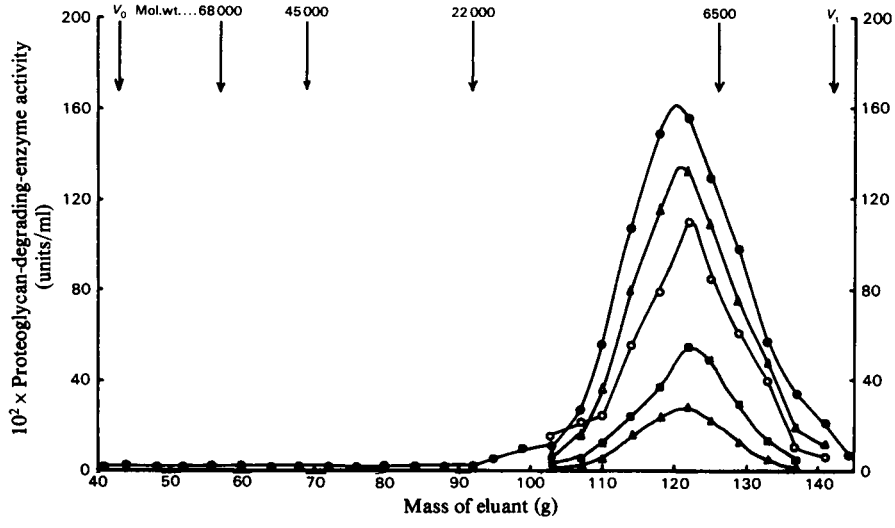

Fig. 5. *Chromatography on Ultrogel AcA 54 of proteinase extracted from synovial-fluid cells and the effect of proteinase inhibitors on the activity*

Washed synovial-fluid cells (1.40×10^7) were extracted with 3 ml of 10 mM-citric acid for 16 h at 4°C and the insoluble residue was removed by centrifugation. The supernatant was adjusted to pH 7.4 with Tris base and a portion (1.0 ml, 15.2 enzyme units) was fractionated on the column system described in the legend to Fig. 2. Portions (50 μl) of each fraction were assayed for activity without further treatment (●) and in the presence of 1 mM-4-chloromercuribenzoate (△), 10 mM-EDTA (○), soya-bean trypsin inhibitor (200 μg/ml) (■) and 2 mM-phenylmethanesulphonyl fluoride (▲). The recovery of activity from the column was 265%.

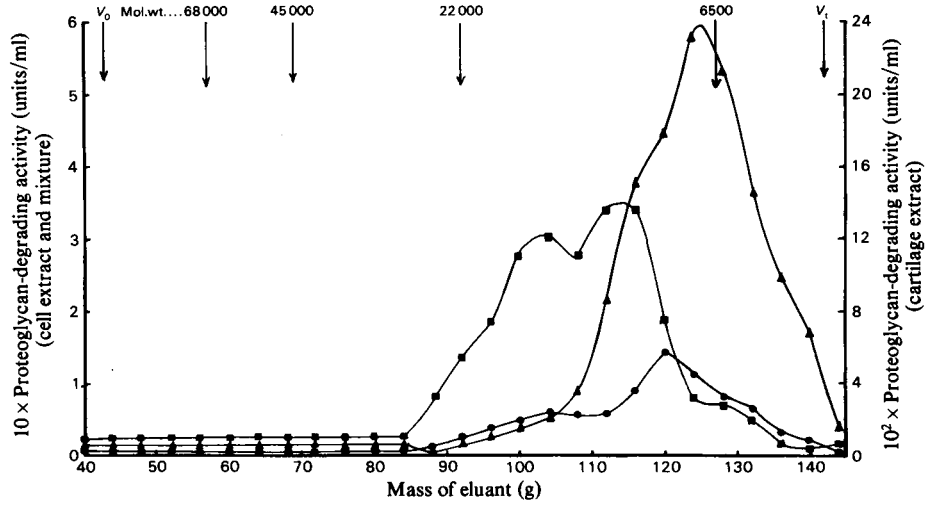

Fig. 6. *Chromatography on Ultrogel AcA 54 of a mixture of cartilage extract and cell extract*
Samples of guanidine hydrochloride extract of arthritic cartilage (1.5 ml, 0.46 enzyme unit) and a citric acid extract of synovial-fluid cells (0.5 ml, 6.86 enzyme units) were fractionated separately, and after mixing, on the column system described in the legend to Fig. 2. Portions (100 μl) of each fraction were assayed for proteoglycan-degrading activity. ●, Cartilage extract; ▲, cell extract; ■, mixture of cartilage extract and cell extract.

(Fig. 3). Also the effect of pH and buffer composition on activity was similar for the two enzyme preparations (Fig. 4).

This similarity in properties of the two enzyme preparations was consistent with the cartilage enzyme being derived from the synovial-fluid cells. However, the elution profile of the cartilage enzyme (Fig. 2) showed an active species eluting between 90 and 110 g that was absent from the cell extract (Fig. 5). The similarity in inhibitor sensitivity of all fractions obtained on chromatography of the cartilage extract (see Fig. 2) indicated that a single enzyme species was present and that the spread in elution behaviour might be due to interaction of this enzyme with other components of the extract.

Evidence in keeping with this was obtained when portions of cartilage extract (0.46 enzyme unit) and cell extract (6.86 enzyme units) were fractionated on Ultrogel AcA 54 separately and after mixing (Fig. 6). As expected, the major peak of activity in the individual samples was at about 125–130 g. However, the activity in the mixture eluted much earlier over a wide range between 90 and 125 g; this suggests that the enzyme in the cell extract can associate with components of the cartilage extract, resulting in elution of the enzyme at a much higher apparent molecular weight.

Studies on the source of the enzyme in synovial-fluid-cell extracts

Although the enzyme in synovial-fluid-cell extracts (Fig. 5) might have been derived from any of the different cell types present, we had previously shown that rabbit polymorphonuclear leucocytes isolated from inflammatory exudates contain high proteoglycan-degrading activities (Welton *et al.*, 1977). Moreover, a neutral proteinase with similar properties had also been described in lysosomal preparations from rabbit leucocytes (Davies *et al.*, 1971; Dewald *et al.*, 1975; Werb *et al.*, 1978). To investigate this possibility, lysosomal granules were isolated from synovial-fluid cells by differential centrifugation (see under 'Methods'), and a citric acid extract of granules (5.4 units/10^7 cells) was fractionated on Ultrogel AcA 54. The activity was recovered at a position ($K_{av.} = 0.78$) virtually identical with that obtained by direct extraction of the mixed cell preparation. Indeed, very similar activity profiles were also obtained on fractionation of two other preparations derived from polymorphonuclear leucocytes: firstly, a citric acid extract of granules isolated from leucocytes of a glycogen-induced peritoneal exudate, and secondly the products secreted by leucocytes (4.5×10^7 cells) in the presence of cytochalasin B, cyclic GMP and heat-aggregated rabbit γ-globulin (see under 'Methods'). These results were generally consistent with the synovial-fluid-cell enzyme (Fig. 5), being derived largely, if not entirely, from polymorphonuclear leucocytes.

Conclusions

The work described demonstrates that short-term joint inflammation results in the accumulation in

articular and meniscal cartilages of a proteinase that degrades proteoglycan at neutral pH. The enzyme isolated from the cartilage exhibited 'elastase-like' properties, in that it was markedly inhibited by serine-proteinase inhibitors and totally inhibited by the specific elastase inhibitor NAc-Ala_2-Pro-Val-CH_2Cl. The enzyme was also highly retarded on Ultrogel AcA 54, consistent with the known non-ideal behaviour of 'elastase-like' enzymes on gel chromatography. Thus human spleen elastase is anomolously retarded on Sephadex G-75 even with high salt concentrations in the eluting buffer (Starkey & Barrett, 1976).

Although the cellular source of this enzyme remains to be proven, all of its properties were very similar to that of the 'elastase-like' enzyme prepared from rabbit polymorphonuclear leucocytes. Both enzymes had an apparent molecular weight on Ultrogel AcA 54 of about 8000 and both showed similar sensitivity to a range of proteinase inhibitors, including a cytosolic inhibitor prepared from rabbit leucocytes. Both enzymes exhibited the same requirements for salt concentration, pH and buffer composition.

If indeed the enzyme in cartilage extracts is derived from leucocytes, the question arises as to its mode of entry into the cartilage. The finding that the synovial fluid apparently contains an excess of proteinase inhibitor suggests that the mechanism does not involve its release from the leucocytes into the synovial fluid. The results would be explained if instead the enzyme was released directly into the articular surface during attempted phagocytosis of immune complexes known to be present in the superficial layers (Cooke *et al.*, 1975; Ishikawa *et al.*, 1975). Such a mechanism, previously suggested to explain cartilage degradation in human rheumatoid arthritis (Barrett, 1978), is supported by the detailed studies on leucocyte enzyme secretion from several laboratories (Henson, 1971; Weissmann *et al.*, 1972; Ignarro, 1974*b*; Weissmann, 1977).

The high enzyme activity present in cartilage from all areas (Table 1) is consistent with the finding that extensive loss of proteoglycan is observed in all areas of articular cartilage in experimental arthritis (Lowther & Gillard, 1976). Indeed the amount of extractable enzyme, about 6–14 units/100mg wet weight, if distributed evenly throughout the cartilage, would be sufficient to degrade the total cartilage proteoglycan of the hind joint of the rabbit in about 24h. The finding that only about 25% of the cartilage proteoglycan is lost during 72h of inflammation (Lowther *et al.*, 1978) indicates that diffusion of the enzyme is restricted *in vivo* or that the enzyme is partly complexed by endogenous inhibitors (Knight *et al.*, 1979). It seems unlikely, however, that this enzyme would be totally inhibited *in vivo*, and detected only after dissociation of enzyme and inhibitor in guanidine hydrochloride; thus low but significant amounts of enzyme were recovered by extraction of cartilage in 0.2M-NaCl, a solvent unlikely to dissociate enzyme–inhibitor complexes.

Calculations based on the results given indicate that, in antigen-induced arthritis of 72h duration, as much as 10% of the total 'elastase-like' activity of the joint polymorphonuclear leucocytes may accumulate within the articular cartilage. Although the involvement of enzymes from other sources cannot be excluded, it would appear that leucocyte-derived enzymes alone may be sufficient to explain the extent of cartilage proteoglycan depletion seen in early rheumatoid arthritis (Janis & Hamerman, 1969). Indeed, such leucocyte elastase may also be involved in the initial stages of cartilage collagen degradation in inflammatory joint disease (Starkey *et al.*, 1977).

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References

Barrett, A. J. (1966) *Nature (London)* **211**, 1188–1189
Barrett, A. J. (1975) in *Dynamics of Connective Tissue Macromolecules* (Burleigh, P. M. C. & Poole, A. R., eds.), pp. 189–226, North-Holland, Amsterdam
Barrett, A. J. (1978) *Agents Actions* **8**, 11–18
Cohn, Z. A. & Hirsch, J. G. (1960) *J. Exp. Med.* **112**, 983–1004
Cooke, T. D., Hurd, E. R., Ziff, M. & Jasin, H. E. (1975) *J. Exp. Med.* **135**, 323–338
Davies, P., Rita, G. A., Krakauer, K. & Weissmann, G. (1971) *Biochem. J.* **123**, 559–569
Dewald, B., Rindler-Ludwig, R., Bretz, V. & Baggiolini, M. (1975) *J. Exp. Med.* **141**, 709–723
Dingle, J. T. (1979) *Ann. Rheum. Dis.* **38**, 201–214
Dingle, J. T., Blow, A. M. J., Barrett, A. J. & Martin, P. E. N. (1977) *Biochem. J.* **167**, 775–785
Dulbecco, R. & Vogt, M. (1954) *J. Exp. Med.* **99**, 167–182
Dumonde, D. C. & Glynn, L. E. (1962) *Br. J. Exp. Pathol.* **43**, 373–383
Hanks, J. H. & Wallace, R. E. (1949) *Proc. Soc. Exp. Biol. Med.* **71**, 196–200
Henson, P. M. (1971) *J. Immunol.* **107**, 1535–1546
Higuchi, Y., Honda, M. & Hayashi, H. (1975) *Cell. Immunol.* **15**, 100–108
Ignarro, L. J. (1974*a*) *Arthritis Rheum.* **17**, 25–36
Ignarro, L. J. (1974*b*) *J. Immunol.* **113**, 298–308
Ignarro, L. J., Oronsky, A. L. & Perper, R. J. (1973) *Clin. Immunol. Immunopathol.* **2**, 36–51
Ishikawa, H., Smiley, J. D. & Ziff, M. (1975) *Arthritis Rheum.* **18**, 563–576
Janis, R. & Hamerman, D. (1969) *Bull. Hosp. Joint Dis.* **30**, 136–152
Knight, J. A., Stephens, R. W., Bushell, G. R., Ghosh, P. & Taylor, T. R. F. (1979) *Biochim. Biophys. Acta* **584**, 304–310

Lowther, D. A. & Gillard, G. C. (1976) *Arthritis Rheum.* **19**, 769–776
Lowther, D. A., Sandy, J. D., Santer, V. B. & Brown, H. L. G. (1978) *Arthritis Rheum.* **21**, 675–680
Powers, J. C., Gupton, B. F., Harley, A. D., Nishino, N. & Whitley, R. J. (1977) *Biochim. Biophys. Acta* **485**, 156–166
Sandy, J. D. (1979) *Biochem. J.* **177**, 569–574
Sandy, J. D., Brown, H. L. G. & Lowther, D. A. (1978) *Biochim. Biophys. Acta* **543**, 536–544
Sandy, J. D., Brown, H. L. G. & Lowther, D. A. (1980) *Arthritis Rheum.* **23**, 433–447
Starkey, P. M. & Barrett, A. J. (1976) *Biochem. J.* **155**, 255–263
Starkey, P. M., Barrett, A. J. & Burleigh, M. C. (1977) *Biochim. Biophys. Acta* **483**, 386–397
Weissmann, G. (1977) *Arthritis Rheum.* **20** (*Suppl.*), S193–S202
Weissmann, G., Spilberg, I. & Krakauer, K. (1969) *Arthritis Rheum.* **12**, 103–116
Weissmann, G., Zurier, R. B. & Hoffstein, S. (1972) *Am. J. Pathol.* **68**, 539–559
Weissmann, G., Goldstein, I., Hoffstein, S. & Tsung, P. K. (1975) *Ann. N. Y. Acad. Sci.* **253**, 750–762
Welton, R. L., Sriratana, A., Neville, R. E. & Lowther, D. A. (1977) *Proc. Aust. Biochem. Soc.* **10**, 4
Werb, Z., Dingle, J. T., Reynolds, J. J. & Barrett, A. J. (1978) *Biochem. J.* **173**, 949–958