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IDENTIFICATION OF PROTEINASES IN RHEUMATOID SYNOVIUM

DETECTION OF LEUKOCYTE ELASTASE CATHEPSIN G AND ANOTHER SERINE PROTEINASE

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Summary

Extracts of rheumatoid synovial tissue obtained at surgical synovectomy contained neutral proteinases as well as cathepsin D. The neutral proteinase activity was particle-bound but could be solubilized by 1 M $MgCl_2$. About half of the solubilized activity adsorbed to aproptinin-Sepharose at pH 7.5 and was desorbed at pH 3.3. This activity was shown to be due to leukocyte elastase and cathepsin G by enzymological and immunological criteria. The neutral proteinase activity that did not adsorb to aprotinin-Sepharose was not due to elastase or cathepsin G. It was able to hydrolyse proteoglycan and was inhibited by diisopropylfluorophosphate, soybean and lima bean trypsin inhibitors. It was, therefore, a serine proteinase. Its inhibition characteristics were different from those of plasmin, kallikrein or thrombin. All of the neutral proteinase activity of synovial extracts was attributable to serine proteinases, no evidence of metallo-proteinases was found. The possible role of the neutral proteinases in the degradation of the matrix of cartilage is discussed.

A simple procedure for purifying leukocyte elastase and cathepsin G is described as well as the raising of specific antisera to these enzymes.

Introduction

In this paper we describe an attempt to identify the neutral proteinases contained in rheumatoid synovium. The histological appearance of the

Abbreviations: Bz-DL-Phe-2-ONap, *N*-benzoyl-DL-phenylalanine 2-naphthyl ester; Tos-Lys-CH_2Cl, tosyllysylchloromethane; Tos-Phe-CH_2Cl, tosylphenylalanylchloromethane; Z-Ala-2-ONap, *N*-benzyloxycarbonyl-L-alanine-2-naphthyl ester.

rheumatoid joint suggests that the synovium eats its way into the articular cartilage, and because of this it has long been thought that proteinases from the synovium degrade the proteoglycan and collagen which form the matrix of the cartilage. Much of the detailed work on this possibility has concentrated on enzymes produced by synovial tissue and cells in culture, with special emphasis on rheumatoid synovial collagenase [1,2]. Cathepsin D (a lysosomal acid proteinase) has been found in rheumatoid synovium [3] and more recently the localization of this enzyme was studied immunocytochemically [4]. The role of cathepsin D in the degradation of the matrix of cartilage is doubtful, because the initial stages of degradation are likely to be occurring extracellularly and, therefore, near to pH 7.4. Cathepsin D has no detectable catalytic activity at or above pH 7 on proteoglycan [5]. It is more likely that neutral proteinases mediate extracellular degradation of the matrix. Earlier workers [6,7] have demonstrated that extracts of rheumatoid synovium showed proteolytic activity at near neutral pH but the enzymes responsible were not characterized or identified.

The work reported in this paper was designed to establish whether or not neutral proteinases (in the sense of enzymes having a pH optimum at about physiological pH) were really detectable in rheumatoid synovial tissue, and if so to isolate and identify them.

Materials and Methods

Benzamidine, Bz-DL-Phe-2-ONap, Tos-Lys-CH_2Cl, Tos-Phe-CH_2Cl, soybean trypsin inhibitor (Type 1-S), lima bean trypsin inhibitor and bovine trypsin (Type 1) were from Sigma (U.K.) Ltd. Aprotinin (Trasylol®) was a gift from Dr. E. Philip, Bayer AG, D-5600 Wuppertal 1, F.R.G. Z-Ala-2-ONap was given by Dr. G.C. Knight of the Strangeways Laboratory. 4-Aminophenylmercuric acetate was from Aldrich Chemical Co., Brij-35 from Fisons and pepstatin from the Peptide Research Foundation, Tokyo, Japan. ^{35}S as sulphate (25—40 Ci/mg) was from The Radiochemical Centre, Amersham, U.K. Sepharose 4B was from Pharmacia, and CM- and DEAE-celluloses (CM52 and DE52) from Whatman Biochemicals.

Preparation of extracts of rheumatoid synovium. Synovium was obtained from patients with definite or classical rheumatoid arthritis (A.R.A. Criteria) who were undergoing surgical synovectomy of the knee joint. The patients were receiving a variety of non-steroidal anti-inflammatory drugs at the time of operation. The specimens were washed in phosphate-buffered saline (137 mM NaCl, 25. mM KCl, 1.1 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) and the synovial lining was dissected away from underlying fibrous and fatty tissue. The lining tissue was weighed and minced as finely as possible with scissors. The mince was washed in three changes of phosphate-buffered saline and suspended in 4 vols. of 0.1 M Tris-HCl buffer, pH 7.5, containing 0.05% NaN_3. This suspension was homogenized by use of an Ultraturrax homogenizer, intermittently, for a total of 5 min. The homogenate was stirred overnight, then strained through gauze, and finally centrifuged at $500 \times g$ for 10 min. The pellet was discarded, and the crude extract of rheumatoid synovium stored at 4° C until it was used (within 24 h). All the procedures were carried out at 4° C.

Azocasein assay. Neutral proteinase activity was measured with azocasein as substrate [8]. Azocasein was chosen since it is a good substrate for many endopeptidases. Reaction mixtures (1.25 ml) contained 0.25 ml 0.4 M Tris-HCl buffer, pH 7.5, 0.25 ml 6% (w/v) azocasein and 0.75 ml of extract. The mixtures were incubated at 50° C for 4 h. The reaction was stopped by adding 1.25 ml of 6% (w/v) trichloroacetic acid and the mixture was filtered. The absorbance of the trichloroacetic acid-soluble reaction products was measured at 366 nm and the ΔA_{366} value was calculated by subtraction of blank values. One unit of enzymic activity was defined as that amount which hydrolyzed 1 mg of azocasein per h under the assay conditions described.

To study the effect of pH on hydrolysis of azocasein the following buffers were used: pH 5.5, 0.4 M sodium acetate; pH 6, 0.4 M sodium maleate; pH 7.0—8.5 0.4 M Tris-HCl; pH 9—10.5, 0.4 M glycine-NaOH.

Assays of inhibitory activity were performed by pre-incubating the inhibitor under test with the enzyme solution for 2 h at 20° C before the substrate was added. Tos-Phe-CH_2Cl was dissolved in propan-2-ol at 10 mM concentration, other inhibitors were dissolved in the assay buffer. For monitoring the purification of elastase and cathepsin G, the azocasein assay was used exactly as described by Starkey and Barrett [8]. The main difference from the assay above being the presence of high salt (1 M KCl) and 0.1% Brij-35 in the incubation mixture.

Esterase assays for elastase and cathepsin G. The substrate for elastase was *N*-benzyloxycarbonyl-L-alanine 2-naphthyl ester (Z-Ala-2-ONap) and for cathepsin G was *N*-benzoyl-DL-phenylalanine 2-naphthyl ester (Bz-DL-Phe-2-ONap). Both were used as described elsewhere [8].

Hydrolysis of proteoglycan. ^{35}S-labelled proteoglycan aggregate was made from bovine nasal cartilage and was entrapped in polyacrylamide beads. The rate of release of radioactivity from the beads at pH 7.5 was used as a measure of hydrolysis of the proteoglycan. The preparation and use of the beads were as described by Dingle et al. [5] except that the volume of the assay was scaled down to 0.25 ml. The reaction was carried out in 0.1 M Tris-HCl, pH 7.5, and the assay tubes were incubated at 37° C for 2 h. ^{35}S which was released from the beads was measured in a liquid scintillation counter. Assays for inhibitory activity were performed by adding substrate after preincubation of the enzyme and inhibitor at 20° C for 2 h. An approximately logarithmic dose-response curve was obtained as described previously [5]. A standard curve was constructed with bovine trypsin (0.01—1.0 μg) for purposes of comparison.

Affinity chromatography. Sepharose 4B was activated with CNBr [9] and coupled to aprotinin in 0.1 M $NaHCO_3$ at pH 8.5. The coupling mixture contained 4 mg of aprotinin/g of activated gel. About 85% of the aprotinin became linked to the gel, and the final preparation bound about 1 mg of trypsin/ml. The aprotinin-Sepharose was used in chromatography columns for experiments with material from synovial extracts. Samples of synovial extracts were applied to columns (1 ml bed volume) of aprotinin-Sepharose equilibrated with 4 mM Tris-HCl buffer, pH 7.5, containing 1 M NaCl and 0.1% Brij-35. Fractions (1-ml) were collected as the columns were washed with 10 vols. of the Tris-HCl buffer. Protein that had adsorbed to the gel bed was eluted with 50 mM glycine-HCl buffer, pH 3.3, containing 1 M NaCl and 0.1% Brij-35.

An immunosorbent for leukocyte elastase was prepared by coupling rabbit antibodies to Sepharose. IgG was prepared by making an $(NH_4)_2SO_4$ fraction (0—50% saturation) from the antiserum (see below), and chromatographing this on DEAE-cellulose in 25 mM Tris-HCl buffer, pH 8.0. The IgG was eluted with a linear gradient of increasing buffer concentration up to 150 mM, and coupled to Sepharose 4B exactly as described for aprotinin except that the coupling buffer was 50 mM sodium phosphate, pH 6.5.

Purification of leukocyte elastase and cathepsin G. This was based on the use of affinity chromatography as described by Baugh and Travis [10]. Leukocytes obtained by leukapheresis from a patient with chronic myeloid leukaemia were a gift from Dr. J. Bradwell, Immunodiagnostic Laboratory, The Medical School, Birmingham, U.K.

The cells (about 70 g) had been washed and were supplied deep-frozen; they were thawed and suspended in 9 vols. of 0.1 M Tris-HCl buffer, pH 7.5, containing 1 M $MgCl_2$ and 0.1% Brij-35. The mixture was homogenized (Ultra-turrax) and then centrifuged at 100 000 × g for 1 h. 95% of the proteinase activity was extracted into the supernatant. Since the supernatant was rather viscous it was diluted with 2 vols. of 5 mM Tris-HCl buffer, pH 7.5, containing 1 M NaCl and 0.1% Brij-35, to a protein concentration of 1—2 mg/ml.

The diluted extract was adsorbed batch-wise with aprotinin-Sepharose, the gel slurry was washed well with the 5 mM Tris-HCl buffer and the proteinases were eluted from the gel with the 50 mM glycine-HCl buffer, pH 3.3 (the same buffers that were used for affinity chromatography of synovial extracts). 100-ml of gel adsorbed about 80 mg of leukocyte proteinases and the same gel could be used repeatedly to process a large amount of material.

The eluates were dialysed against 50 mM sodium phosphate buffer, pH 6.0, and batches of 150 mg of protein were applied to a carboxymethyl cellulose (Whatman CM 52) column (25 ml bed volume) equilibrated with the same buffer. A linear gradient (1 l) to 0.8 M NaCl in the buffer eluted elastase at about 0.3 M NaCl and cathepsin G at about 0.6 M. Since the peak of elastase tailed into that of cathepsin G, the contaminated cathepsin G was passed through the immunosorbent for leukocyte elastase. This step successfully removed the elastase (as judged by Ouchterlony immunodiffusion). The specific activity of different batches of purified enzymes on azocasein ranged from 310—325 units/mg for elastase and 330—360 units/mg for cathepsin G. Both enzymes were pure as judged by electrophoretic criteria. Protein concentration was calculated from an $A^{1\%}_{1cm}$ value at 280 nm of 9.85 [10] for elastase and 6.67 for cathepsin G (our value). Enzyme purity was assessed by electrophoresis on SDS 10% polyacrylamide gels [11] and on polyacrylamide gels run at acid pH [12].

Antisera and immunodiffusion. Antiserum to elastase was raised in rabbits by giving four subcutaneous injections of 200 μg of protein in complete Freund's adjuvant at monthly intervals. A total of four injections were given. Antiserum to cathepsin G was produced by injecting guinea-pigs according to a similar schedule. Attempts to produce a cathepsin G antiserum in rabbits were unsuccessful.

Before they were mixed with adjuvant, both enzymes were inactivated by incubating them with 1 mM diisopropylfluorophosphate. The unreacted inhibi-

tor was removed by dialysis after an overnight incubation at 4° C.

Ouchterlony gel diffusion plates were made from 1% agar in phosphate buffer containing 1 M NaCl. Antisera were tested against leukocyte proteinases and leukocyte extracts which had first been inactivated with diisopropylfluorophosphate. The active proteinases gave spurious precipitin lines when tested against non-immune serum, and these were not seen when inactivated enzymes were used. Each antiserum gave a single precipitin line against a leukocyte extract, the line being identical to that given by the purified antigen. There was no precipitin line when the anti-elastase serum was tested against cathepsin G or vice versa.

Results

Detection and solubilization of neutral proteinase activity

Fig. 1 shows a typical curve for the pH dependence of hydrolysis of azocasein by an extract of rheumatoid synovium. The activity below pH 7 was attributed to cathepsin D since it was inhibited by pepstatin. When the assays were performed in the presence of pepstatin, a broad pH optimum was seen in the pH range 7—9. This corresponded to the shoulder of the pH-dependence curve obtained in the absence of pepstatin. The hydrolysis of azocasein at pH 7.5 was linear with respect to time of incubation and amount of extract added up to $\Delta A_{366} = 0.4$. Inclusion of 1 M KCl in the assay mixture (at pH 7.5) caused 40—50% inhibition, and 0.1% Brij-35 caused 20—30% increase in activity. When the extract was centrifuged at 100 000 × *g* for 1 h 80—85% of the activity at pH 7.5 sedimented. The pellet was frozen and thawed (to solubilize cathepsin D), washed by resuspending it in the 0.1 M Tris-HCl buffer and recentrifuged. The pH dependence of this particulate preparation is shown in Fig. 1. It is almost the same as that obtained for the crude extract in the presence of pepstatin. It was concluded that the shoulder on the pH-dependence curve was due to neutral proteinase activity which was largely particle-bound. The activity was solubilized by adding $MgCl_2$ to the washed pellet to 1 M final

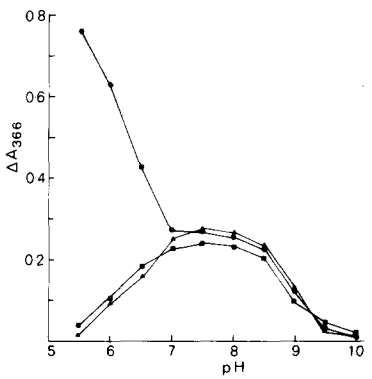

Fig. 1. Dependence on pH of proteolytic activity of rheumatoid synovial extract. Assays were performed with azocasein substrate in buffers specified in the text. ●———●, crude synovial extract; ▲———▲, crude synovial extract in the presence of pepstatin (10 μg/ml); ■———■, 100 000 × *g* pellet of a crude synovial extract which had been freeze-thawed, washed and recentrifuged as described in the text.

concentration and then recentrifuging at 100 000 × *g* for 1 h. After this treatment over 90% of the proteinase recovered was in the supernatant, and the overall recovery of activity was 80%. $MgCl_2$ was more effective than KCl, which at 2 M gave only 60% solubilization.

$MgCl_2$ extracts were made from samples of six rheumatoid synovia (5—14 g wet wt.). The homogenates were centrifuged at 500 × *g* for 10 min and the pellets discarded. The supernatants were centrifuged at 100 000 × *g* for 1 h and the pellets were resuspended to the original volume in the buffer used for homogenization. After recentrifugation the pellets were suspended in half the volume of buffer containing 1 M $MgCl_2$, and centrifuged again. The supernatants were saved and contained 1—2 units of azocasein hydrolysing activity per g (wet wt.) of synovial tissue use.

Affinity chromatography

The $MgCl_2$ extracts were dialysed against the 5 mM Tris-HCl buffer and passed down columns of aprotinin-Sepharose. Bound proteinase was eluted with the 50 mM glycine-HCl buffer, pH 3.3. Extracts from six different synovia were chromatographed and 32—68% (mean 46%) of the activity was adsorbed and eluted at acid pH. The remainder of the activity passed straight through the columns. Overall recoveries of activity from the columns was 75—85%.

Detection of leukocyte elastase and cathepsin G

Since the leukocyte proteinases, elastase and cathepsin G, were adsorbed by aprotinin attached to Sepharose, it was thought that the proteinase activity of the synovial extracts which had adsorbed to the affinity columns might be attributable to these enzymes. The material which had been eluted from the affinity columns at acid pH was assayed for activity against Z-Ala-2-ONap (a substrate for elastase) and Bz-DL-Phe-2-ONap (a substrate for cathepsin G). Calibration curves were constructed for each substrate with pure elastase or cathepsin G, and the amount of each enzyme present in the samples was calculated. From these figures it was possible to calculate the amount of azocasein hydrolysis expected from the sample. Table I shows the results obtained

TABLE I

PROTEINASE ACTIVITY IN SYNOVIAL SAMPLES ELUTED FROM APROTININ-SEPHAROSE COLUMNS AT pH 3.3

Elastase and cathepsin G activities were measured by using the naphthyl esters, Z-Ala-2-ONap and BZ-DL-Phe-2-ONap. The calculated azocaseinase values were derived from the figures obtained in the naphthyl esterase assays and were based on the specific activities of the batches of purified enzymes used to calibrate the esterase assays. The assays were performed on fractions with maximal azocasein activity. If the fractions were pooled the activity ratios (elastase:cathepsin G:azocaseinase) were the same.

	Elastase (μg/ml)	Cathepsin G (μg/ml)	Calculated azocaseinase (units/ml)	Observed azocaseinase (units/ml)
Syn 1	1.23	0.98	0.72	0.85
Syn 3	5.4	3.8	3.0	3.4
Syn 4	1.37	0.816	0.69	0.73
Syn 5	0.1	6.0	2.1	2.3
Syn 6	1.6	1.75	1.1	1.7

for material from five synovial extracts. The material from synovia 1, 3, 4 and 6 contained elastase and cathepsin G in similar amounts, whereas synovium 5 contained little elastase activity but more cathepsin G than the other samples. The amount of azocasein hydrolysis calculated from the esterase assays and the observed activity on azocasein are also shown.

The predicted and observed activities were close, except for synovium 6. Since the esterase assays were not necessarily specific for elastase and cathepsin G, the material desorbed from the affinity columns was also tested by Ouchterlony gel diffusion against specific antisera to the enzymes. Fig. 2a shows that the rabbit antiserum to the leukocyte elastase gave a single precipitin line when tested against the crude leukocyte extract and that the line was identical with that given by the pure enzyme. Fig. 2b shows a single precipitin line between the synovial samples and the antiserum; the line appears

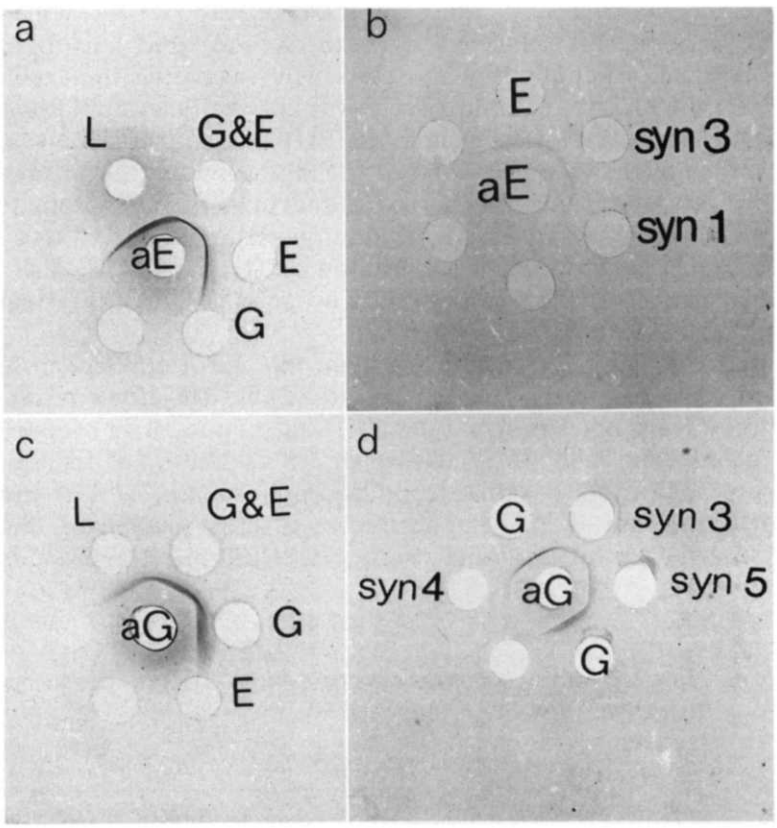

Fig. 2. Demonstration by Ouchterlony gel diffusion of leukocyte elastase and cathepsin G in samples purified from rheumatoid synovium by affinity chromatography. The synovial samples were those assayed for elastase and cathepsin G after being purified on aprotinin-Sepharose (Table I). (a) aE, rabbit antiserum to leukocyte elastase, 20 μl of $\frac{1}{4}$ dilution; L, 20 μl of crude $MgCl_2$ extract of leukocytes; G, cathepsin G, 1 μg; E, leukocyte elastase, 1 μg; G and E, 1 μg of each proteinase. (b) aE, antiserum to elastase, 20 μl of 1/32 dilution; E, leukocyte elastase, 100 ng; syn 3, sample from synovium 3, 10 μl; syn 1, sample from synovium 1, 40 μl. (c) aG, guinea-pig antiserum to cathepsin G, 20 μl; otherwise as for a. (d) aG, guinea-pig antiserum to cathepsin G, 20 μl of $\frac{1}{4}$ dilution; G, cathepsin G, 500 ng; syn 3, and syn 5, 20 μl of synovial samples; syn 4, 40 μl of synovial sample.

identical to that against purified leukocyte elastase. Fig. 2c and d show similar experiments in which the guinea-pig antiserum against cathepsin G was used. These results confirmed that the synovial proteinases desorbed from the aprotinin-Sepharose were leukocyte elastase and cathepsin G. The close agreement between the predicted and observed values for hydrolysis of azocasein makes it likely that these were the only proteinases desorbed from the aprotinin-Sepharose.

Properties of the neutral proteinase that did not absorb to aprotinin-Sepharose

As described earlier, 30—70% of the neutral proteinase of the synovial extracts showed no affinity for the aprotinin-Sepharose. When the material was tested in gel diffusion there was no precipitin line with either of the antisera against the leukocyte enzymes. Table II shows results of inhibition assays of the activity obtained with samples from four synovia. The results for the different synovia are similar: there was almost complete inhibition with diisopropylfluorophosphate, showing that the proteinase(s) was of the serine-catalytic class. The activity was inhibited by both soybean and lima bean trypsin inhibitors. It was not inhibited by aprotinin, which was consistent with it failing to adsorb to the affinity column. There was no significant inhibition by Tos-Lys-CH_2Cl, Tos-Phe-CH_2Cl or benzamidine. EDTA gave 40% inhibition of the material from synovium 1, but in view of the inhibition by diisopropylfluorophosphate, the activity can safely be attributed to a serine proteinase rather than to a metallo-proteinase. The inhibition spectrum of this activity does not correspond to thrombin (which is inhibited by Tos-Lys-CH_2Cl), kallikrein (inhibited by aprotinin) or plasmin (inhibited by Tos-Lys-CH_2Cl, soybean trypsin inhibitor and aprotinin).

The material that had passed straight through the aprotinin-Sepharose columns also hydrolysed the proteoglycan substrate. Table III shows results obtained with material from one synovial sample. 10 μl (about 15 μg protein) of the sample gave the same amount of release of ^{35}S as 120 ng of trypsin. There was complete inhibition by diisopropylfluorophosphate, which was strong evidence for the release of ^{35}S being caused by a serine proteinase. The degree of inhibition by soybean and lima bean trypsin inhibitors represents

TABLE II

INHIBITION CHARACTERISTICS OF NEUTRAL PROTEINASE OF SYNOVIAL EXTRACTS AFTER PASSAGE THROUGH APROTININ-SEPHAROSE COLUMNS TO REMOVE ELASTASE AND CATHEPSIN G

Values expressed as % inhibition of activity in the azocasein assay. Dip-F, diisopropylfluorophosphate.

Inhibitor	Concentration	Syn 1	Syn 2	Syn 3	Syn 4
Dip-F	1 mM	75	84	90	95
EDTA	2 mM	40	20	9	15
Tos-Lys-CH_2Cl	1 mM	12	0	0	0
Tos-Phe-CH_2Cl	0.2 mM	12	10	20	17
Benzamidine	2 mM	8	0	15	6
Soybean trypsin inhibitor	100 μg/ml	86	91	93	95
Lima bean trypsin inhibitor	100 μg/ml	86	93	83	95
Aprotinin	100 μg/ml	0	0	9	8

TABLE III

EFFECT OF VARIOUS AGENTS ON THE HYDROLYSIS OF ^{35}S-PROTEOGLYCAN SUBSTRATE BY SYNOVIAL NEUTRAL PROTEINASE

10 μl of synovial material which had not adsorbed to aprotinin-Sepharose were used in each bead assay (0.013 azocasein units). The control release of ^{35}S (200 cpm) has been subtracted. The uninhibited enzyme released about 25% of the total ^{35}S in the beads. Dip-F, diisopropylfluorophosphate.

Inhibitor or activator	Concentration	cpm released/100 μl assay mixture
None	—	600
Dip-F	1 mM	11
EDTA	2 mM	152
Soybean trypsin inhibitor	100 μg/ml	151
Lima bean trypsin inhibitor	100 μg/ml	52
Aprotinin	200 μg/ml	658
Aminophenylmercuric acetate	0.5 mM	553

about 90% inhibition when estimated as the amount of active enzyme on the semi-log standard curve [5]. There was no evidence of activation in the presence of aminophenylmercuric acetate, such as might have been expected if a latent metallo-proteinase were present [13]. Attempts to purify the enzyme(s) responsible for this activity have so far been unsuccessful. It adsorbed to lima bean and soybean trypsin inhibitors attached to Sepharose, but the binding was strong and it was not possible to elute active enzyme under conditions of mild alkaline or acidic pH. Gel filtration of the material in the presence of high-salt concentration and detergent gave a single broad protein peak in the eluate with the enzyme distributed throughout, and ion-exchange chromatography on carboxymethyl cellulose was similarly unsuccessful.

Discussion

The validity of the identification of leukocyte proteinases in the synovial tissue extracts depends on the purity of our leukocyte enzyme preparations. The method we have used to purify the enzymes was convenient in that it was not necessary to make a granule preparation from the leukocytes, so that deep-frozen cells could be used. We relied on ion-exchange chromatography to separate the two proteinases, and the cathepsin G from this step contained only 2—3% elastase. However, it was necessary to remove this in order to raise a specific antiserum to cathepsin G. The enzymes were electrophoretically pure and gave electrophoresis patterns similar to previous preparations in this and other laboratories [8,10]. The specific activity of the elastase (allowing for the different expression of units) was about the same, and that of the cathepsin G rather higher, than previously [8]. The overall purification from whole cells was about 30-fold for both enzymes, and similar quantities of each were obtained (200 mg) from starting material containing about 20 g of protein.

The results showed that, on average, about half of the neutral proteinase activity that could be extracted from rheumatoid synovium was due to the leukocyte proteinases, elastase and cathepsin G. As far as is known these two enzymes occur only in the polymorphonuclear leukocyte [14], a cell not commonly seen in the rheumatoid synovium, but abundant in rheumatoid

synovial fluid. Some authors have stressed the absence of polymorphonuclear leukocytes from the rheumatoid synovial pannus [15,16], although in a recent study by Mohr and Wessinghage [17] polymorphonuclear leukocytes were identified by means of a histochemical stain for esterase activity (naphthol-AS-D-chloroacetate) * and were shown to be present at the pannus/cartilage interface. Kobayashi and Ziff [18] also observed polymorphonuclear leukocytes in rheumatoid pannus in what they called 'cell-nests', which were groups of cells apparently penetrating the cartilage. In view of the ability of leukocyte elastase and cathepsin G to attack both the proteoglycan and collagen of cartilage [19,20], and the large amount of the enzymes in the cells (at least 5% of the cell protein), these enzymes may well participate in the cartilage destruction apparently caused by pannus. We cannot be sure that the leukocyte proteinases we have identified biochemically are from intact polymorphonuclear leukocytes in the tissue. They may be from discharged leukocyte granules, or even from some other cell type. We hope that immunohistochemical studies may provide evidence as to their source.

About half of the neutral proteinase activity from the synovia was not attributable to the leukocyte proteinases, thrombin, plasmin or kallikrein. We have been unable to detect any neutral serine proteinases other than elastase and cathepsin G in peripheral blood leukocytes and all of the detectable serine proteinase activity of leukocyte extracts adsorbed to aprotinin-Sepharose. The unidentified synovial activity must therefore have been due to enzymes from some other cell type. Diisopropylfluorophosphate, soybean and lima bean trypsin inhibitors inhibited this activity on both azocasein and proteoglycan, suggesting that it was the same enzyme(s) acting on both substrates. The enzyme(s) may have come from macrophages or lymphocytes which are present in rheumatoid synovium. A neutral serine proteinase has been found in rat macrophages [21] and a surface-associated serine proteinase has been reported in human monocytes [22]. Human lymphocytes contain neutral proteolytic activity [23] and a 'cytotoxic' proteinase has been purified from them by affinity chromatography on STI-Sepharose [24]. This must be a different enzyme from the synovial one since we were unable to elute active enzyme from soybean trypsin inhibitor-Sepharose columns.

We were unable to detect any metallo-proteinase even in the presence of aminophenylmercuric acetate. Thus, we conclude that all of the neutral proteinase in the extracts of rheumatoid synovium that hydrolysed azocasein or proteoglycan was attributable to enzymes of the serine-catalytic class. Further work is needed on their distribution before their role in matrix degradation can be assessed.

Since our studies have been confined to rheumatoid tissue we are unable to say whether neutral serine proteinases are present in normal synovial tissue. As the cellular composition of normal and rheumatoid synovium is so different (because of the inflammatory reaction in the synovium) such a comparison would be of dubious value. Furthermore, measurements of levels of enzymes in crude tissue extracts are difficult to interpret because of the presence of inhibitors and other substances which may affect the results of the assays.

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* Naphthol-AS-D-chloroacetate, 2-(chloroacetyl)-3-naphthoic acid ortho-toluidide.

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