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STUDIES ON THE EFFECT OF PENTOSAN POLYSULFATE ON PROTEOGLYCAN DEGRADATION BY LEUKOCYTE NEUTRAL PROTEASES

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Abstract—Pentosan polysulfate (SP-54), an anti-inflammatory agent, inhibits the degradation of proteoglycan by a leukocyte neutral protease preparation enriched with elastase. Under the assay conditions used, a 50 per cent inhibition is observed at approximately 3.5×10^{-8} M pentosan polysulfate. Pentosan polysulfate, at concentrations up to 1×10^{-5} M, does not affect the proteolytic degradation of proteoglycan catalyzed by trypsin, chymotrypsin and pancreatic elastase. The inhibitory effect of pentosan polysulfate is most probably related to the interaction of the drug with the proteoglycan substrate. The resulting complex is stable to proteolytic degradation by leukocyte neutral proteases. This complex is readily dissociated by 0.5 M sodium chloride and the resulting proteoglycan can be subsequently degraded by the leukocyte protease preparation.

A characteristic feature of inflammatory joint disease, such as rheumatoid arthritis, is an infiltration of PMN§ into the joint space. The granule fraction of these cells has been shown to contain at least two neutral proteases, elastase and cathepsin G, that are capable of degrading the proteoglycans of the cartilage matrix [1–6]. A variety of mechanisms have been reported to account for the release of these proteases from the cells [7]. The net result of this event is the degradation of cartilage proteoglycans and the loss of metachromasia. The view that neutral proteases of PMN contribute to tissue damage is supported by the obversation that intra-articular administration of these enzymes into the joints of normal rats results in cartilage and bone damage and an arthritic-like syndrome [8].

Although the etiology of the inflammation in joint diseases is complex and mediated by several events [9], it appears that the cartilage and bone damage that occurs in the disease is, at least in part, due to the neutral proteases released from the PMN. Drugs that specifically inhibit the release or the activity of these enzymes should be of therapeutic value in the treatment of the disease. A variety of anti-inflammatory drugs have been tested *in vitro* for their effects on PMN neutral proteases [10, 11]. Extending these earlier observations [10, 11], the present *in vitro* studies indicate that pentosan polysulfate, an anti-inflammatory agent [12], is a potent inhibitor of proteoglycan degradation by PMN elastase. The specificity of this effect and the mechanism of action of pentosan polysulfate are reported.

MATERIALS AND METHODS

Human leukocytes enriched with PMN were isolated by dextran sedimentation [13]. The leukocytes were suspended in 100 mM Tris–HCl, pH 7.4, containing 5 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.05% Triton X-100. The cells were disrupted by sonication and, after centrifugation at 27,000 *g* for 20 min, the clear supernatant fluid was fractionated with ammonium sulfate. The protein fraction precipitating between 30 and 70% saturation with ammonium sulfate was isolated, suspended in 20 mM Tris–HCl, pH 7.4, and used as the source of crude PMN neutral proteases.

Proteoglycans labeled with ^{35}S were prepared by the method of Dingle *et al.* [14]. Briefly, 22 g of cleaned and diced bovine nasal septum (used within 3 hr of sacrifice) was incubated with 150 ml of culture medium (GIBCO—F-13 Joklik Modified supplemented with antibiotics and glutamine) containing 25 mCi of $^{35}SO_4$ (New England Nuclear Corp., Boston, MA.). After 18 hr at 37°, the labeled cartilage was isolated by filtration and washed extensively with F-13 medium. ^{35}S-Labeled proteoglycans were extracted as described by Dingle *et al.* [14].

Cross-linked polyacrylamide beads containing entrapped ^{35}S-labeled proteoglycans were prepared by the method of Dingle *et al.* [14]. The specific activity of the polyacrylamide beads containing the labeled proteoglycans was approximately 1.5×10^4 cpm of ^{35}S/mg of dry beads.

Assays for proteoglycan degrading PMN neutral proteases. The assay system in a total volume of 1.0 ml contained 100 mM Tris–HCl (pH 7.4), 2.0 mg (dry weight) of polyacrylamide beads containing ^{35}S-labeled proteoglycan (3×10^4 cpm of ^{35}S) and aliquot of the PMN neutral protease preparation. After 30 min at 37° in a shaking water bath, the assay mixture was cooled to 4°. The cooled reaction mixture was centrifuged to pellet the polyacrylamide beads and an aliquot of the clear supernatant fluid was assayed for radioactivity. Reaction mixtures that did not contain the protease preparation were used as blanks.

The activity of the neutral proteases on the degradation of free ^{35}S-labeled proteoglycan was also deter-

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§ The following abbreviations have been used: PMN, polymorphonuclear leukocytes; TCA, trichloroacetic acid; CPC; cetylpyridinium chloride; *N*-acetyl AAPVCK, *N*-acetyl alanylalanyl prolyl valyl chloromethylketone; PMSF, phenylmethyl sulfonyl fluoride; SP-54, pentosan polysulfate; and EEDQ, *n*-ethoxycarbonyl 2-ethoxy 1, 2-dihydroquinoline.

mined by the method of Sapolsky *et al.*[15]. Approximately 3×10^4 cpm of free [^{35}S] proteoglycan was incubated in 100 mM Tris–HCl, pH 7.4, as above. After 30 min at 37°, CPC was added to the reaction mixture to precipitate the intact and degraded proteoglycans. The precipitate was collected by centrifugation and the degraded proteoglycans were selectively solubilized by the addition of TCA [15]. The TCA soluble material was assayed for radioactivity. Reaction mixtures devoid of the neutral protease preparation served as blanks.

Human PMN elastase was purified by the procedure of Baugh and Travis [16]. This preparation was homogenous by the criteria of gel electrophoresis.

Pentosan polysulfate (SP-54) was obtained from Bene-Chemie, Munich, Germany. PMSF was obtained from CalBiochem, LaJolla, CA and *N*-acetyl AAPVCK was a gift from Dr. James Powers, Georgia Institute of Technology, Atlanta, GA. EEDQ is a product of the Aldrich Chemical Co., Milwaukee, WI and trasylol was a generous gift of Bayer, Wuppertal, W. Germany. Pancreatic elastase was obtained from Worthington Biochemicals, Freehold, NJ.

RESULTS

Studies by Dingle *et al* [14] have shown that polyacrylamide beads containing entrapped^{35}S-labeled proteoglycan can be used as substrate in developing a sensitive assay procedure for analyzing proteoglycan degrading enzymes. In this assay, the degraded proteoglycan diffuses out of the beads and is measured by radio-chemical analysis of the supernatant fluid. As shown in Fig. 1, the release of the ^{35}S-labeled material from the beads was linear up to 30μg of protein in the PMN neutral protease preparation. Degradation of the labeled proteoglycan was completely inhibited by the addition of PMSF (5×10^{-3} M) to the reaction mixture. Addition of *N*-acetyl AAPVCK (1×10^{-4}M) resulted in 60–80 per cent inhibition of ^{35}S-release, suggesting that the preparation was highly enriched in elastase. This preparation was not inhibited by the addition of EEDQ (1×10^{-4} M), indicating the absence of cathepsin G in the preparation.

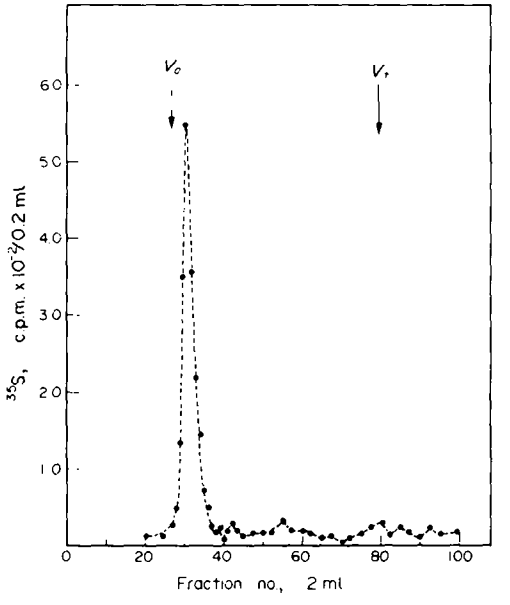

Fig. 2. Sephadex G-100 chromatography of the degraded [^{35}S]proteoglycan released from [^{35}S]proteoglycan entrapped in polyacrylamide beads.

The inhibition of release of ^{35}S by PMSF and by *N*-acetyl AAPVCK indicates that the enzymatic release of ^{35}S from the beads is primarily due to elastase and not due to a sulfatase or glycosidase activity present in the preparation.

Evidence that the ^{35}S-released material was large in size was obtained by chromatography of the released material on Sephadex G-100. A sample containing 3.2×10^4 cpm of the ^{35}S-labeled product (released from the beads after digestion by PMN proteases) was concentrated by ultrafiltration (PM-10) and layered on a Sephadex G-100 column (1.5 x 91 cm). The column was developed with 50 mM Tris–HCl, pH 7.4. Approximately 70 percent of the applied radioactivity eluted near the void volume of the column (Fig. 2). No radioactivity could be detected in the other fractions.

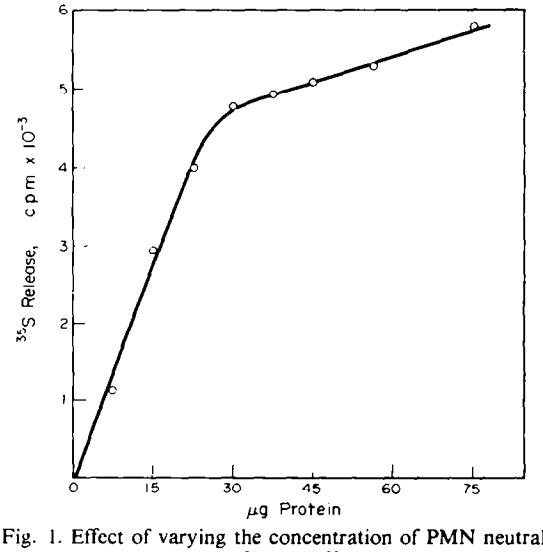

Fig. 1. Effect of varying the concentration of PMN neutral proteases on the release of ^{35}S from ^{35}S-labeled proteoglycan entrapped in polyacrylamide beads.

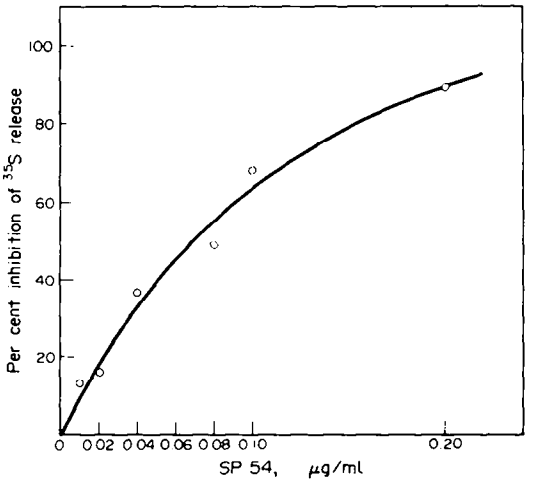

Fig. 3. Effect of varying the concentration of pentosan polysulfate (SP-54) on [^{35}S]proteoglycan degradation by PMN neutral proteases.

Table 1. Effect of pentosan polysulfate on proteoglycan degradation by purified PMN elastase*

Pentosan polysulfate (μg)	^{35}S-released (cpm)	Inhibition (%)
0	3896 ± 300	0
0.1	3038 ± 310	22
0.2	2103 ± 220	46
0.4	1246 ± 120	68
1.0	287 ± 30	94

* Thirteen μg of protein in the purified elastase preparation was used. Other details of the assay are described in the text. Results represent the average of three assays.

The effect of pentosan polysulfate on the activity of PMN neutral protease preparation was examined (Fig. 3). Addition of pentosan polysulfate (SP-54) to the assay mixture resulted in a dose-dependent inhibition of proteoglycan degradation (^{35}S-release) with a 50 percent inhibition at approximately 0.07 μg/ml. Assuming an average molecular weight of 2000 for pentosan polysulfate, a 50 percent inhibition is observed at approximately 3.5×10^{-8} M.

Since a major portion of the proteolytic activity in the crude preparation was due to elastase, the effect of pentosan polysulfate on purified PMN elastase was examined (Table 1). Addition of pentosan polysulfate to the reaction mixture resulted in a dose–dependent inhibition of elastase activity, and 50 percent inhibition was observed at approximately 0.1 μM.

The effect of pentosan polysulfate on the degradation of proteoglycans by other proteolytic enzymes was examined (Table 2). Under conditions in which pentosan polysulfate inhibited the degradation by PMN neutral proteases, the drug had no effect on trypsin, chymotrypsin or pancreatic elastase-dependent activity. These observations suggest that the inhibition by pentosan polysulfate of proteoglycan degradation by PMN neutral proteases may be fairly specific.

Mechanism of action of pentosan polysulfate. Preincubation of the PMN neutral protease preparation with pentosan polysulfate did not inactivate the proteolytic activity. In these experiments, 30 μg of protein in the protease preparation was preincubated in 0.1 ml of 100 mM Tris–HCl, pH 7.4, with varying concentrations of pentosan polysulfate (0.1 to 0.5 μg/ml). After 15 min at 37°, 0.01-ml aliquots of the preincubation mixture were assayed for residual protease activity. Under conditions where 0.1 μg/ml of pentosan polysulfate in the final assay mixture (Fig. 3) showed greater than 60 percent inhibition, exposure of the enzyme preparation in the preincubation to 0.1 μg/ml of pentosan polysulfate showed no appreciable inactivation (less than 5 percent). With higher concentrations of pentosan polysulfate, the extent of inhibition was due to the carryover of the pentosan polysulfate from the preincubation to the assay mixture. If the pentosan polysulfate had interacted with the enzyme, preincubation of the enzyme with the drug should have resulted in loss of enzymatic activity. This assumption is based on the premise that the enzyme–pentosan polysulfate complex, if formed, is stable and does not dissociate during the bead assay. The view that the drug was interacting with the proteoglycan substrate was supported by the following experiments: 30 mg (dry weight) of [^{35}S]proteoglycan polyacrylamide beads in 3 ml of 20 mM Tris–HCl buffer, pH 7.4, was preincubated at 37° for 15 min with 30 μg pentosan polysulfate. A reaction mixture incubated as above but without the pentosan polysulfate was used as a control. Both reaction mixtures were centrifuged and an aliquot of the beads (representing 2 mg dry weight) was tested for its ability to serve as a substrate for the PMN neutral proteases. The enzymatic release of ^{35}S from the beads that had been preincubated with pentosan polysulfate was inhibited approximately 80 percent when compared to the control. These observations indicate that

Table 2. Effects of pentosan polysulfate on proteoglycan degradation by PMN neutral proteases, trypsin, chymotrypsin and pancreatic elastase*

Enzyme	Pentosan polysulfate (μg)	^{35}S-released (cpm)	Inhibition (%)
PMN neutral proteases	0	4431 ± 400	0
	0.05	2658 ± 262	40
(30 μg protein)	2.0	300 ± 32	93
	20.0	222 ± 30	95
Crystalline trypsin	0	1437 ± 140	0
	2.0	1497 ± 140	0
(2 μg)	20.0	1419 ± 140	0
Crystalline chymotrypsin	0	1563 ± 160	0
	2.0	1467 ± 150	6
(2 μg)	20.0	1419 ± 150	9
Crystalline pancreatic	0	1608 ± 170	0
elastase	2.0	1638 ± 185	0
(2 μg)	20.0	1561 ± 160	3

* Details of the assay are described in the text. Results represent the average of three assays.

pentosan polysulfate interacts with the proteoglycan polyacrylamide beads and the resulting complex is relatively stable to degradation by PMN netural proteases.

The dissociation of the complex was studied. The beads that had been preincubated with pentosan polysulfate were collected by centrifugation and divided into two aliquots. One half of the beads was washed four times each with 10 ml of 20 mM Tris–HCl, pH 7.4. The other half of the beads was washed as above in 20 mM Tris–HCl, pH 7.4, containing 0.5 M NaCl. After each wash, an aliquot of the beads (representing 2 mg dry weight) was used as a substrate for PMN neutral proteases. As stated above, beads that had been preincubated with pentosan polysulfate were relatively stable to degradation by PMN neutral proteases (Fig. 4). When these beads were assayed without washing (0 wash), the enzymatic release of ^{35}S was inhibited by about 80 percent when compared to control. Sequential washing of the beads with 20 mM Tris–HCl, pH 7.4, did not modify this inhibitory effect. However, if these beads were washed in buffer containing 0.5 M NaCl, the ^{35}S-release from the washed beads approached that of control beads. These observations support the view that the complex resulting from the interaction of pentosan polysulfate and proteoglycan polyacrylamide beads dissociates readily in buffers of high ionic strength.

The possibility that pentosan polysulfate may be binding to the polyacrylamide of the [^{35}S]proteoglycan polyacrylamide beads and interfering with the degradation of [^{35}S]proteoglycan was examined. Free [^{35}S]proteoglycan was incubated with PMN neutral proteases at 37° for 30 min in the presence of varying concentrations of pentosan polysulfate. At the end of the incubation, the amount of degraded [^{35}S]proteoglycan was measured by the method of Sapolsky *et al.*[15]. The result of this experiment is shown in Table 3. Inhibition of proteoglycan degradation by pentosan polysulfate was also observed using this assay method. A 50 percent inhibition of proteoglycan degradation by PMN neutral protease was obtained at approximately 0.15 μg/ml of pentosan polysulfate. The above observation indicates that the inhibition of proteoglycan degradation by pentosan polysulfate is not due to an interaction of the drug with polyacrylamide, but is related to its interaction with [^{35}S]proteoglycan.

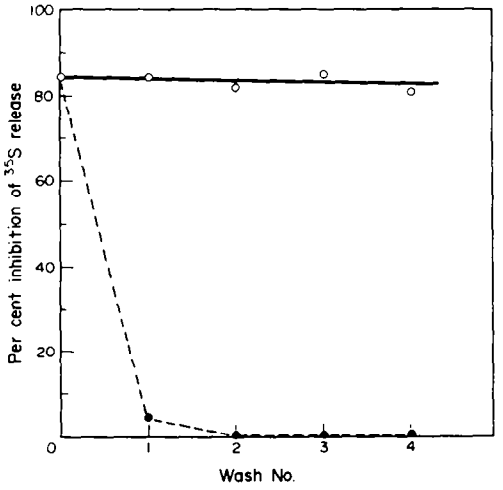

Fig. 4. Dissociation of the pentosan polysulfate–[^{35}S]proteoglycan polyacrylamide complex. Key: (○—○) complex washed in 20 mM Tris–HCl, pH 7.4, and (●—●) complex washed in above buffer containing 0.5 M sodium chloride.

Table 3. Effects of pentosan polysulfate on the degradation of free proteoglycan by PMN neutral proteases*

Pentosan polysulfate (μg)	^{35}S-released (cpm)	Inhibition (%)
0	4224 ± 430	0
0.05	3678 ± 380	13
0.10	2514 ± 260	39
0.20	1347 ± 140	68
1.0	495 ± 50	89

* Thirty μg of protein in the PMN neutral protease preparation was used. Other details of the assay are described in the text.

DISCUSSION

Pentosan polysulfate, a semisynthetic polysulfated xyloside, is a potent anti-inflammatory and anti-complement agent [11, 17]. The present studies indicate that this sulfated polysaccharide is a potent inhibitor of proteoglycan degradation by PMN elastase. Pentosan polysulfate does not inhibit the activity of pancreatic elastase. The observation that pentosan polysulfate inhibits proteoglycan degradation by PMN elastase but has no effect on pancreatic elastase is somewhat surprising. However, apparent differences between these enzymes may be the reason and such differences have been reported [18]. For example, Starkey and Barrett [18] have compared the effects of various inhibitors on lysosomal and pancreatic elastase and their studies suggest that the susceptibility to inhibition of these enzymes by inhibitors is different.

Studies on the mechanism of action of this drug suggest that it binds to the proteoglycan substrate, and the resulting complex is stable to proteolysis by PMN neutral proteases. If this were to occur *in vivo*, the half-life of pentosan polysulfate in the joint, when administered intra-articularly, would be considerably extended due to its interaction with proteoglycans of the cartilage matrix. Furthermore, the drug should protect the proteoglycans of the cartilage matrix from tissue damage by PMN elastase present in the synovium.

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