[Chem. Pharm. Bull. 24(2) 215—219 (1976)]

UDC 546.41-38.49.09 : 547.478.6.09 : 615.27.071.9

Inhibitory Effect of D-Penicillamine on Degradation of Hexosamine-containing Substances in the Involution of Carrageenin Granuloma induced by Calcium Chelate Ethylenediaminetetraacetate[1]

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(Received May 15, 1975)

The effect of calcium chelate ethylenediaminetetraacetate (Ca-EDTA) and D-penicillamine on the content of hexosamine-containing substances in the carrageenin granuloma in rats was tested by means of their daily local injection into pre-formed granuloma pouch during 3 or 4 days.

The administration of Ca-EDTA revealed involution of pre-formed granuloma and significantly decreased the content of glycosaminoglycans (especially, hyaluronic acid and chondroitin sulphate A) and glycoproteins. However, when Ca-EDTA and D-penicillamine were simultaneously administered or D-penicillamine was given 8 hr after the injection of Ca-EDTA, involution of granulomatous tissue and degradation of both hexosamine-containing substances were completely inhibited. Free N-acetyl-β-glucosaminidase activity in granuloma also increased by treatment with Ca-EDTA, while free enzyme activity recovered to the control level by simultaneous treatment with Ca-EDTA and D-penicillamine. These results obtained here were discussed in relation to the mode of action of D-penicillamine as an antirheumatic drug.

Recently, D-penicillamine has been used in the treatment of rheumatoid arthritis, particularly because of its effect on the rheumatoid factor. However, mode of action of D-penicillamine in this disease has not yet been clarified in all its details.

Injection of carrageenin solution into subcutaneous tissue induces a complex reaction characterized first by inflammation and deposition of connective tissue, and later by resorption of the entire process.[3-6] This biphasic reaction has been used as a model of subacute inflammation and proliferative inflammation.[7,8] Recent papers demonstrated that betamethasone disodium phosphate can induce the involution of pre-formed carrageenin granuloma[8] and also the decrease of hexosamine-containing substances in granulomatous tissue, while D-penicillamine was ineffective.[9]

On the other hand, Chvapil[10] demonstrated that the administration of a high dose of calcium chelate ethylenediaminetetraacetate (Ca-EDTA) to rats caused a marked increase in the urinary excretion of hydroxyproline, but it was suppressed by zinc acetate or dexamethasone. He considered that the lysosomal membrane labilized by Ca-EDTA was stabilized by zinc acetate or dexamethasone.

In the rheumatoid arthritis lysosomal membrane is labilized and the degradation of connective tissue occurred. Therefore, in the present study, we have investigated the influence

1) A part of this work was presented at the 95th Annual Meeting of the Pharmaceutical Society of Japan, Mukogawa, April 1975.
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of D-penicillamine on the content of glycosaminoglycans and glycoproteins in the granulomatous tissue of rats after treatmentwith Ca-EDTA, known as the unstabilizer of lysosomal membrane.

Materials and Methods

Treatment of Animals——Carrageenin granulomas were induced in male rats of Donryu strain weighing about 120 g, according to the method described by Fukuhara and Tsurufuji.[8] The day of carrageenin injection was designated as day 0. All the animals received laboratory rat chow (CLEA Inc., Tokyo, Japan) and tap water throughout the experiments. Drugs used were D-penicillamine (Taisho Pharmaceutical Co., Ltd, Tokyo, Japan) and Ca-EDTA. Ca-EDTA was prepared according to the method of Aronson and Rogerson.[11] Aqueous solution of Ca-EDTA (45 mg/rat/day) and D-penicillamine (60 mg/rat/day) in saline were simultaneously administered into the carrageenin granuloma pouch from day 6 up to day 8, while control animals were given saline only (Experiment I). In another experiment, D-penicillamine (60 mg/rat/day) solution was injected into the pouch 8 hr after the injection of Ca-EDTA (45 mg/rat/day) solution into the pouch from day 6 to day 9 (Experiment II). These animals were killed on day 9 or 10 by severing the carotid artery. Besides the control killed on day 9 or 10 (9- or 10-day control), a group of rats were killed on day 6 just before the treatment of drugs was started (6-day control). The entire fluid in the granuloma pouch was harvested, then the capsule of granulomatous tissue was removed carefully. "Final body weight" was calculated by subtracting "pouch fluid" and "pouch wall" from the gross body weight.

Isolation of Glycosaminoglycans and Glycoproteins——The granulomatous tissue was minced, dehydrated, and defatted with several changes of acetone and $CHCl_3$–MeOH (1 : 1, v/v) mixture. The dry weight of granuloma was measured desiccating the tissue over P_2O_5. The dry tissue (150—200 mg) was digested with pronase E (2 mg/10 mg dry tissue, Kaken Co., Tokyo, Japan) for 48 hr. The digest was added with trichloroacetic acid to 10% concentration and the precipitated protein was centrifuged off. The supernatant was dialyzed against running tap water for 48 hr and against distilled water for successive 24 hr. The dialysate was evaporated *in vacuo* and NaCl was added to 0.04 M concentration. Crude cetylpyridinium-glycosaminoglycan complex was precipitated by adding 1% cetylpyridinium chloride (CPC). After extracting the supernatant with amyl alcohol at 0°, the aqueous layer was dialyzed against running tap water for 48 hr and against distilled water for successive 24 hr. The dialysate was evaporated in a rotary evaporator. This fraction was designated as glycoproteins. The cetylpyridinium-glycosaminoglycan complex was dissolved in 1 ml of 4M NaCl, precipitated with 14 ml of 80% ethanol twice. The glycosaminoglycans were separated into "non-sulphated" and "sulphated" components by CPC-Celite method according to the method previously described by Schiller, *et al.*[12] It was already confirmed in a previous work[9] that "non-sulphated glycosaminoglycan" was hyaluronic acid (HA) and "sulphated glycosaminoglycans" consisted of chondroitin sulphate A (ChS-A), chondroitin sulphate B (ChS-B) and heparan sulphate (HS) in granuloma of both 6- and 10- day controls.

Analytical Methods——Hexosamine was determined by a modification of the Elson-Morgan reaction[13] after hydrolysis of the sample in 2N HCl at 100° for 16 hr. Sialic acid analysis was performed by thiobarbituric acid method according to the Warren's method[14] after hydrolysis in 0.05 M H_2SO_4 at 80° for 1 hr.

Electrophoresis on Cellulose Acetate Membrane——The glycosaminoglycans fractionated were subjected to electrophoresis on cellulose acetate membrane with 0.3M calcium acetate at 1 mA/cm for 3 hr according to the method of Seno, *et al.*[15] To stain the glycosaminoglycans, 1% Alcian Blue in 2% AcOH was used.

Analysis of Chondroitin Sulphate Isomers——Quantitative analysis of chondroitin sulphate isomers was performed by the chondroitinase method of Yamagata *et al.*[16] Hexosamine derivatives was determined by a modification of the Morgan-Elson reaction.[17]

Isolation of Granuloma Cells Free from Intercellular Matrix——The granulomatous tissue was cut into small pieces and dispersed with a mixture of 0.05% collagenase from Clostridium hystolyticum (Sigma Chemical Co., St. Louis, Mo.U.S.A., Type I) and 0.05% trypsin (Difco Lab. Detroit, Mich.U.S.A.) in phosphate-buffered saline at 37° for 40 min. After vigorous pipetting, the dispersed cells were filtered through a coarse surgical gauze and collected by centrifugation (600 g, 5 min). The cells were washed once with Ham's medium containing 10% fetal bovine serum. The final cell pellet was resuspended in 5 ml of SVE medium (medium containing 0.25M sucrose, 1 mM Versene, and 0.1% ethanol), and was disrupted with 25 strokes of a motor-

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driven glass-Teflon homogenizer. The homogenate was quantitatively transferred to a conical tube and centrifuged at 600 g for 5 min. The postnuclear supernatant (PNS) was removed with a Pasteur pipette and kept at 4°.

Latency of N-Acetyl-β-glucosaminidase——One-half milliliter of suitably diluted PNS was incubated for 30 min at 37° with 0.5 ml of 1 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide (BDH chemicals Ltd., Poole, England) in 0.25M sucrose containing 0.05M citrate buffer (pH 4.5), with (total activity) or without (free activity) 0.1% Triton X-100. Measurement of *p*-nitrophenol produced was made by spectrophotometry at 400 nm. Latency of the enzyme is shown as the difference between total and free activity and expressed as bound activity of the enzyme.

Results and Discussion

As shown Table I, wet and dry weight of granulomatous tissue of rats treated with Ca-EDTA were significantly lower than that of control. Administration of Ca-EDTA revealed involution of pre-formed granuloma and significantly decreased the content of glycosaminoglycans and glycoproteins as indicated in Table II. However, when Ca-EDTA and D-penicillamine were administered at the same time or D-penicillamine was given 8 hr after Ca-EDTA treatment, the involution of granulomatous tissue was completely inhibited and the content of both hexosamine-containing substances recovered to the control level. Analysis of the glycosaminoglycans showed that HA decreased significantly, whereas the sulphated glycosaminoglycans were unaffected by the treatment of Ca-EDTA. As indicated in Table III, the ratio of HA in total glycosaminoglycans from granuloma of 10-day control was about 58%, but the ratio of HA decreased to about 38% by Ca-EDTA treatment. The composition of sulphated glycosaminoglycans in granuloma was examined using chondroitinase method and the results are given in Table IV. The ratio of ChS-A to ChS-B in the Ca-EDTA-treated group was 30 to 70, while the ratio in 10 day control was 50 to 50. These results indicated that the Ca-EDTA treatment reduced not only HA content, but also ChS-A content, and D-penicillamine completely inhibited the decrease of these glycosaminoglycans. Futhermore, matrix free cells were isolated from 10 day granulomatous tissue and lysosomal N-acetyl-β-glucosaminidase activity, related to degradation of hexosamine-containing substances was assayed. The results are shown in Table V. Free enzyme activity was increased by treatment with Ca-EDTA, while it was recovered to the control level by D-penicillamine treatment.

TABLE I. The Effect of Ca-EDTA and D-Penicillamine on the Pre-formed Carrageenin Granuloma in Rats

Group	No. of rats	Final body weight (g)	Pouch fluid (ml)	Pouch wall	
				Wet weight (g)	Dry weight (g)
Exp. I					
6-Day control	5	162±11	12±2	5.42±0.23	0.456±0.033
9-Day control	5	173± 5	22±3	4.91±0.21	0.471±0.029
D-Penicillamine	5	173± 8	25±5	4.73±0.34	0.445±0.022
D-Penicillamine+Ca-EDTA	5	152± 6	27±4	4.72±0.22	0.457±0.026
Ca-EDTA	5	143± 9	18±4	2.90±0.52[a)]	0.320±0.027[a)]
Exp. II					
6-Day control	5	154± 6	8±2	3.84±0.20	0.408±0.026
10-Day control	4	173± 6	23±8	4.16±0.23	0.545±0.027
D-Penicillamine	5	174± 6	20±3	3.72±0.27	0.569±0.037
D-Penicillamine+Ca-EDTA	4	152± 9	23±4	3.73±0.30	0.513±0.026
Ca-EDTA	5	130±11	28±3	3.14±0.15[a)]	0.434±0.019[b)]

All data are shown as means±S.E.
significantly different from 10 day control (*a*) $p < 0.01$, (*b*) $p < 0.02$

Table II. The Effect of Ca-EDTA and D-Penicillamine on the Content of Hexosamine-containing Substances in Carrageenin Granuloma in Rats

Group	No. of rats	Glycosaminoglycans hexosamine (μg)	Glycoproteins	
			Hexosamine (μg)	Sialic acid (μg)
Exp. I				
6-Day control	5	2244±129	3137±174	2977±168
9-Day control	5	1971±222	3158±284	2943±190
D-Penicillamine	5	1645± 94	2812±196	2845±564
D-Penicillamine+Ca-EDTA	5	2021±205	2746±108	2413± 93
Ca-EDTA	5	1049±114[a]	1657±126[a]	1071±167[b]
Exp. II				
6-Day control	5	2403±139[c]	3582±252	2925±195
10-Day control	4	1927±117	4189±256	3333±183
D-Penicillamine	5	1978±195	4031±290	3297±210
D-Penicillamine+Ca-EDTA	4	2082±111	4051±316	3406±321
Ca-EDTA	5	1445± 59[a]	2716± 64[a]	1861± 98[b]

All data are shown as means±S.E.
significantly different from 10 day control (*a*) $p<0.01$, *b*) $p<0.001$, *c*) $p<0.05$)

Table III. The Effect of Ca-EDTA and D-Penicillamine on the Glycosaminoglycan Composition in Carrageein Granuloma in Rats (Exp. II.)

Group	No. of rats	Glycosaminoglycans (μg of hexosamine)		Ratio[a] (%)
		Non-sulphated	Sulphated	
6-Day control	5	1341±102[b]	342± 42	79.7
10-Day control	4	890± 20	632± 69	58.5
D-Penicillamine	5	827± 50	739±100	52.8
D-Penicillamine+Ca-EDTA	4	882± 52	687± 41	56.2
Ca-EDTA	5	432± 35[c]	692± 36	38.4

All data are shown as means±S.E.
a) ratio of non-sulphated glycosaminoglycans to total glycosaminoglycans
significantly different from 10 day control (*b*) $p<0.01$, *c*) <0.001)

Table IV. Relative Proportion of Chondroitin Sulphate Isomers

Group	Chondroitin sulphate (%)		
	A	B	C
6-Day control	70	30	0
10-Day control	49	50	1
D-Penicillamine	49	51	0
D-Penicillamine+Ca-EDTA	49	50	1
Ca-EDTA	30	69	1

Chondroitin sulphate isomers were analysed by the chondroitinase method.

Lysosomal enzymes have been shown to increase dramatically during the resorption of carrageenin granuloma in physiological condition.[18] This is strong presumptive evidence that these enzymes play a major role in this process. In this study, the cell species that release these enzymes are not determined. However, it has already been suggested that macrophages in granulomatous tissue may be damaged by the ingested carrageenin, thereby

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Table V. N-Acetyl-β-glucosaminidase Activity of Cells Isolated from 10 Day Granulomatous Tissue

Group	No. of rats	Bound activity (%)[a]
10-Day control	3	32.8±1.0
D-Penicillamine	3	30.3±0.8[b]
D-Penicillamine+Ca-EDTA	3	30.5±2.5[c]
Ca-EDTA	3	22.2±0.4[d,e]

All data are shown as means±S.E.
a) bound activity (%)=A−*B*/*A*×100, *A*; total activity, *B*; free activity
significantly different from 10-day control (*b*) $p<0.02$, *c*) $p<0.01$, *d*) $p<0.001$), significantly different from D-penicillamine+Ca-EDTA, (*e*) $p<0.05$)

releasing their lysosomal enzymes.[18]. One possibility is that the two drugs used are related to this process. On the other hand, we recently found that the release of N-acetyl-β-glucosaminidase into medium in the cultivation of carrageenin granuloma cells was stimulated by treatment with Ca-EDTA under the condition that was morphologically intact.[19] It is not determined which of the two processes contributes much to the stimulation of resorption of carrageenin granuloma by injection of Ca-EDTA into pouch. On the basis of the results obtained here, it seems reasonable to assume that Ca-EDTA stimulates the release of lysosomal enzymes such as testicular type hyaluronidase at least, and that D-penicillamine inhibits these processes.

Donner, *et al.*[20] reported that ChS-B in the control group rose steadily from 40% of total chondroitin sulfates after 1 month of regeneration to 54% at 4 month, while ChS-B in D-penicillamine-treated rabbits remained constant at about 40% of total in tendon regeneration experiment. In our experiment ChS-B increased only slightly contrary to the above results.

It is well known that HA in synovial fluid was converted into small molecular size and neutral collagenase and lysosomal enzymes were activated in rheumatoid arthritis. These phenomena are considered to be one of factors of this disease. Weissmann[21] emphasized that lysosomal enzymes might be important in the pathogenesis of inflammatory process in the joints of patients with rheumatoid arthritis. However, Anderson[22] reported that D-penicillamine which is known as stabilizer of membrane of isolated liver lysosomes[23] could not inhibit the activation of lysosomal enzymes by adjuvant arthritis.

Present study suggested that the activity of lysosomal enzyme of D-penicillamine group was not different from that of the control, but D-penicillamine completely inhibited the release of lysosomal enzyme such as found in the granuloma treated with Ca-EDTA. These facts suggested that D-penicillamine has been prescribed on an experimental basis in the treatment of patients with rheumatoid arthritis.

Acknowledgement The authors thank Mr. M. Okada for his technical assistance in a part of this study. They also wish to thank Dr. M. Ozeki, Taisho Pharmaceutical Co. Ltd. for a generous gift of D-penicillamine.

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