[Chem. Pharm. Bull. 25(6)1220—1224(1977)]

UDC 615.332.015.4.581.192

Inhibitory Effect of D-Penicillamine on the Fibrosis caused by Bleomycin Treatment in Rat Carrageenin Granuloma[1]

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(Received September 3, 1976)

Administration of bleomycin (1 mg/rat/day) for 5 days into granuloma pouch resulted in significant increase of intercellular substances of the granulomatous tissue measured at the 5th day from the last injection of bleomycin, *i.e.*, 130% for collagen, 261% for non-sulfated glycosaminoglycan, 129% for sulfated glycosaminoglycan and 153% for glycoprotein, while no significant alteration was observed in desoxyribonucleic acid and ribonucleic acid contents.

The increment of these macromolecules caused by bleomycin treatment was inhibited by successive administration of D-penicillamine (60 mg/rat/day) for 5 days.

Keywords——carrageenin granuloma; D-penicillamine; belomycin; glycosaminoglycan; collagen; glycoprotein

Bleomycin, an antitumor antibiotic of glycopeptide isolated from the culture filtrates of *Streptomyces verticillus*,[3,4] has been found to be a most effective agent against several types of cancer,[5] particularly in the treatment of squamous cell carcinomas in the skin and lung. With increasing use, however, it has become apparent that diffuse pulmonary fibrosis occurs as a side effect of bleomycin therapy,[6,7] and measures controlling the side effects of bleomycin is desired. Mice[8,9] and dogs[10] have often been used for the purpose of studying the side effects of bleomycin treatment. However, the methods so far used in these intact animals, have big faults that it takes very long time, more than 30 days in the case of mice, to cause experimental fibrosis by bleomycin treatment, and some superior experimental models have been desired. This report describes firstly our proposed experimental model using rat carrageenin granulomas which is able to cause experimental fibrosis with bleomycin within only 10 days, and secondly concerning the inhibitory effect of D-penicillamine on the fibrosis caused by bleomycin.

Materials and Methods

Treatment of Animals——Carrageenin granulomas were induced in male rats of Sprague-Dawley strain weighing 140—150 g, according to the method described by Fukuhara, *et al.*[11] The day of carrageenin injection was designated as day 0. Bleomycin and D-penicillamine were gifts from Nippon Kayaku Co., Ltd., Tokyo and Taisho Pharmaceutical Co., Ltd., Tokyo, respectively. Aqueous solution of bleomycin

1) A part of this work was presented at the 8th Annual General Meeting of the Japanese Society for Connective Tissue Research, Nagoya, June, 1976.
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3) H. Umezawa, K. Maeda, T. Takeuchi, and Y. Okami, *J. Antibiotics*, (Tokyo), Ser. A, **19**, 200 (1966).
4) H. Umezawa, *J. Jap. Med. Assoc.*, **62**, 144 (1969).
5) Clinical Screening Group, EORTC., *Br. Med. J.*, **2**, 643 (1970).
6) R.A Rudders and G.T. Hensley, *Chest*, **63**, 626 (1973).
7) C.W.M. Bedrossian, M.A. Luna, B. Mackay, and B. Lightiger, *Cancer*, **32**, 44 (1973).
8) I.Y.R. Adamson and D.H. Bowden, *Am. J. Pathol.*, **77**, 185 (1974).
9) M. Ichihashi, H. Shinkai, M. Takei, and S. Sano, *J. Antibiotics*, **26**, 238 (1973).
10) R.W. Fleischman, J.R. Baker, G.R. Thompson, U.H. Schaeppi, V.R. Illievski, D.A. Cooney, and R.D. Davis, *Thorax*, **26**, 675 (1971).
11) M. Fukuhara and S. Tsurufuji, *Biochem. Pharmacol.*, **18**, 475 (1969).

(0.1—1 mg/rat/day) in saline was daily administered into the carrageenin granuloma pouch from day 5 through day 9. Aqueous solution of D-penicillamine (60 mg/rat/day) in saline was daily administered into the carrageenin granuloma pouch from day 10 through day 14. Control animals were given saline only. These animals were killed on day 10 or day 15 by cutting the carotid artery. The exudate filled in the granuloma pouch was carefully collected and then the pouch wall of the granuloma was removed.

Isolation of Acidic glycosaminoglycans and Glycoproteins——The minced tissue was completely dehydrated, and defatted with acetone and mixture of chloroform–methanol (1 : 1, v/v). Acidic glycosaminoglycans and glycoproteins were isolated as described in a previous paper.[12] The acidic glycosaminoglycans were fractionated into "non-sulfated" and "sulfated" components by cetylpyridinium chloride-celite method described by Schiller, *et al.*[13] and also in our previous work.[12]

Analytical Methods——Desoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in the granulomatous tissue were extracted by the method of Schmidt-Thannhauser-Schneider[14,15] and measured by the diphenylamine reaction[16] and the orcinol reaction,[17] respectively.

Hexosamine was determined by a slightly modified method of the Elson-Morgan reaction[18] after hydrolysis of the sample with 2N HCl at 100° for 16 hr.

Hydroxyproline was estimated according to the method of Kivirrikko, *et al.*[19] after hydrolysis of the sample with 6N HCl at 100° for 18 hr.

Results

Effect of Bleomycin on the Formation of Granuloma

The rats were treated with bleomycin at the dose of 0.1 mg or 1 mg/rat/day for 5 days started at the day 5 after carrageenin injection, and killed at day 10 and day 15. The granulomatous tissue was removed and its dry weight was measured. Results are shown in Fig. 1. The dry weight of the granulomatous tissue of the group treated with 1 mg of bleomycin was significantly greater than that of control at day 15, though it was smaller than control at day 10. Therefore, the dose of bleomycin was fixed at 1 mg/rat/day hereafter.

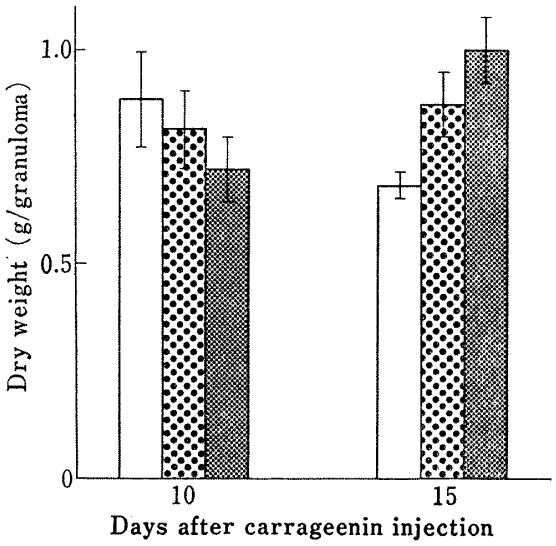

Fig. 1. Effect of Bleomycin on Dry Weight of Carrageenin Granuloma in Rats

Bleomycin was injected daily into granuloma pouch for 5 days from day 5 to day 9.
□: control
▤: bleomycin 0.1 mg,
▩: bleomycin 1 mg

Table I shows the change of various components of the granuloma after treatment with 1 mg/rat/day of bleomycin in the same manner as described above. The wet weight and dry weight of the granulomatous tissue increased during day 5 to day 10 in the control, while they decreased by bleomycin treatment to 61% for wet weight and to 54% for dry weight of the control. Moreover, after withdrawal of bleomycin, the wet weight and dry weight of the granulomatous tissue in bleomycin treated rats increased significantly, *i.e.*, to 127% for wet weight

12) K. Otsuka and Y. Mori, *Chem. Pharm. Bull.* (Tokyo), **24**, 215 (1976).
13) S. Schiller, G.A. Slover, and A. Dorfman, *J. Biol. Chem.*, **236**, 983 (1961).
14) G. Schmidt and S.J. Thannhauser, *J. Biol. Chem.*, **161**, 83 (1945).
15) W.C. Schneider, *J. Biol. Chem.*, **164**, 747 (1946).
16) K. Burton, *Biochem. J.*, **62**, 315 (1956).
17) W.C. Schneider, *Method in Enzymology*, **3**, 680 (1957).
18) R. Gott and E.R. Berman, *Anal. Biochem.*, **15**, 167 (1966).
19) K.I. Kivirrikko, O. Laitinen, and D.J. Prockop, *Anal. Biochem.*, **19**, 249 (1967).

and to 122% for dry weight. As to the contents of DNA and RNA in the granulomatous tissue at day 10, they decreased by bleomycin treatment, but the decrease recovered to the control level in the following 5 days.

TABLE I. Effect of Bleomycin on the Preformed Carrageenin Granuloma in Rats

		Days after carrageenin injection		
		5	10	15
Pouch fluid (g)	control (5)	12.2 ±2.3	36.8 ±3.5	48.0 ±6.1
	bleomycin (4)		10.5 ±5.1	24.5 ±6.7
			$p<0.01$	$p<0.05$
Pouch wall (g) wet weight	control (5)	5.43 ±0.77	8.34 ±0.56	6.37 ±0.40
	bleomycin (4)		5.10 ±0.55	8.07 ±0.63
			$p<0.01$	$p<0.05$
Dry weight	control (5)	0.432±0.069	0.896±0.057	0.829±0.056
	bleomycin (4)		0.483±0.059	1.012±0.044
			$p<0.01$	$p<0.05$
DNA (mg)	control (5)	9.568±1.388	19.69 ±1.29	21.40 ±1.68
	bleomycin (4)		10.12 ±1.77	19.84 ±1.09
			$p<0.01$	N.S.
RNA (mg)	control (5)	18.20 ±3.33	36.26 ±2.08	32.91 ±3.06
	bleomycin (4)		18.38 ±3.90	28.13 ±2.33
			$p<0.01$	N.S.

Results are expressed as mean ± S.E. Bleomycin (1 mg/rat/day) was daily injected into granuloma pouch for 5 days from day 5 to day 9.

In spite of decrease of DNA and RNA as well as wet and dry weight in bleomycin treated granuloma, collagen and hexosamine-containing substances showed an upward tendency even at day 10. (Table II and III) The production rate of these ground substances seemed to be gradually accelerated during bleomycin treatment and increased rapidly after withdrawal of bleomycin. Further fractionation of the hexosamine-containing substances revealed that increase of non-sulfated glycosaminoglycan was the most eminent, *i.e.*, 198% at day 10 and 261% at day 15 (Table III). This non-sulfated fraction was mostly composed of hyaluronic acid as reported previously.[12] These finding that the production of all the intercellular substances including collagen, sulfated and non-sulfated glycosaminoglycans and glycoproteins was significantly enhanced by bleomycin treatment at day 15 strongly suggest the experimental occurrence of fibrosis in the granulomatous tissue.

TABLE II. Effect of Bleomycin on Collagen Contents in Rat Carrageenin Granuloma

	Days after carrageenin injection (mg hydroxyproline/mg DNA)		
	5	10	15
control (5)	0.792±0.125	1.487±0.111	1.300±0.061
Bleomycin (4)		1.694±0.343(114)	1.696±0.036(130)
		N.S.	$p<0.001$

Results are expressed as mean ± S.E.
The percent of control is shown in parenthesis.
Bleomycin (1 mg/rat/day) was daily injected into granuloma pouch for 5 days from day 5 to day 9.

Table III. Effect of Bleomycin on Hexosamine-containing Substances in Rat Carrageenin Granuloma

	Days after carrageenin injection (μg hexosamine/mg DNA)		
	5	10	15
Acidic glycosaminoglycan (AGAG)			
0.5 M NaCl fr. (non-sulfated AGAG)			
Control (5)	132.2±24.5	101.9± 8.1	40.4± 2.9
Bleomycin (4)		201.4±27.1(198)	105.5± 9.0(261)
		$p<0.01$	$p<0.001$
2.1 M NaCl fr. (sulfated AGAG)			
Control (5)	49.5± 6.3	60.7± 4.5	48.9± 3.7
Bleomycin (4)		69.8±11.5(115)	63.0± 3.3(129)
		N.S.	$p<0.05$
Glycoprotein			
Control (5)	451.0±33.0	350.0±20.0	274.0±10.0
Bleomycin (4)		467.0±25.0(133)	419.0±32.0(153)
		$p<0.01$	$p<0.01$

Results are expressed as mean ± S.E.
The percent of control is shown in parenthesis.
Bleomycin (1 mg/rat/day) was daily injected into granuloma pouch for 5 days from day 5 to day 9.

Table IV. Effect of Bleomycin and D-Penicillamine on the Preformed Carrageenin Granuloma in Rats

	Expt.	DNA[a]	Collagen[b]	Acidic glycosaminoglycan[c]		Glycoprotein
				0.5M NaCl fr.	2.1M NaCl fr.	
I	Control (5)	21.46±1.68	27.51±1.43	865± 96	1040± 95	5808±299
	Control+ D-penicillamine (5)	21.80±3.59 N.S.	22.45±3.31 N.S.	882± 84 N.S.	801±175 N.S.	5078±280 N.S.
II	Bleomycin (4)	19.80±3.59	33.62±1.79	2082±161	1252±106	8219±302
	Bleomycin+ D-penicillamine (5)	16.00±2.38 N.S.	23.36±3.03 $p<0.05$	1744±293 N.S.	701±187 $p<0.05$	5971±584 $p<0.02$

a) mg/granuloma
b) mg hydroxyproline/granuloma
c) μg hexosamine/granuloma
Results are expressed as mean ± S.E. Expt. I and II were performed at the same time with the same group of animals.

Inhibitory Effect of D-Penicillamine on the Fibrosis caused by Bleomycin Treatment in Granuloma

Effect of D-penicillamine on the fibrosis caused by bleomycin treatment was studied. D-Penicillamine (60 mg/rat/day) was daily administered for 5 days started from just after stop of bleomycin treatment, *i.e.*, from day 5 through day 9. Experimental design was shown in Chart 1. Then, the contents of DNA, collagen, acidic glycosaminoglycans and glycoproteins in granuloma were compared with those in the control. The results were shown in Table

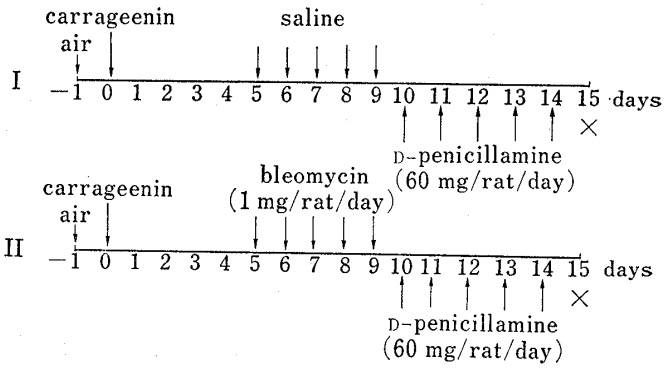

Chart 1. Experimental Design for Table IV

IV. D-Penicillamine itself had no effect on the contents of these macromolecules, but when it was administered to bleomycin treated granuloma, it inhibited the increase of these macromolecules caused by bleomycin treatment.

Discussion

We have previously reported that a culture system of fibroblasts provided a useful *in vitro* tool for studying the mechanism of fibrosis caused by bleomycin, where it was demonstrated that 4 day treatment with 0.1 μg bleomycin/ml resulted in a significant increase in the production of collagen and acidic glycosaminoglycan by a cloned fibroblasts in culture.[20,21] On the other hand, mice have generally been used[8,9] concerning *in vivo* experimental model for the fibrosis caused by bleomycin. In that method, mouse must receive bleomycin daily for 10 days and be matured for another 20 days before getting fibrosis. One of our purposes of this work was to establish another *in vivo* experimental model in which fibrosis took place in a shorter period. Rat carrageenin granuloma was found to answer the purpose. Successive 5 day injection of bleomycin into carrageenin granuloma pouch and another 5 day maturation caused significant increase of collagen, acidic glycosaminoglycan and glycoprotein contents without increasing DNA and RNA contents in the granulomatous tissue. (Table I, II, III) It is noteworthy that the increase of non-sulfated glycosaminoglycan, which was found to be mainly composed of hyaluronic acid in the carrageenin granuloma in our previous experiment,[12] was most evident, *i.e.*, about 200% and 250% at day 10, 15 respectively. The fact is in good agreement with our results obtained from *in vitro* culture system, which showed induced hyaluronic acid synthetase by bleomycin treatment in cultured fibroblasts (the data will be published elsewhere).

A part of this remarkable accumulation of the intercellular substances by bleomycin might be due to decreased degradation, since Ichihashi, *et al.*[9] reported the reduction of cutaneous collagenase activity by bleomycin, and we also found a decline of N-acetyl-β-D-glucosaminidase activity due to bleomycin treatment in cultured fibroblast.[21]

Our next purpose of this work was to discover a suitable treatment for suppressing fibrosis caused by bleomycin. D-Penicillamine was investigated, since studies on the culture of skin from patients with active scleroderma[22] and the rapidly growing tissue such as embryonic rat bone[23] and skin[24] revealed marked decrease of collagen synthesis in D-penicillamine treatment, while in normal rat skin, it was revealed that there was no differences in collagen synthesis between control and D-penicillamine treated cultures.[25] As shown in Table IV, all the intercellular components enhanced by bleomycin decreased significantly by the treatment with D-penicillamine without giving any affection on these components in control tissue. Further investigation on the mechanism of inhibition by D-penicillamine on the biosynthesis of connective tissue components is under way in our laboratory.

Acknowledgement The authers are grateful to Taisho Pharmaceutical Co. and Nippon Kayaku Co. for the generous supplies of D-penicillamine and bleomycin used in this study. They also wish to express their thanks to Mr. A. Fukushima for his experimental work.

20) K. Otsuka, S. Murota, and Y. Mori, *Chem. Pharm. Bull.* (Tokyo), **23**, 3038 (1975).
21) K. Otsuka, S. Murota, and Y. Mori, *Biochim. Biophys. Acta.*, **444**, 359 (1976).
22) C.M. Herbert, K.A. Lindberg, M.I.V. Jayson, and A.J. Bailey, *Lancet*, **1**, 187 (1974).
23) J. Uitto, *Biochim. Biophys. Acta*, **194**, 498 (1969).
24) K. Lindberg, Ph. D. thesis, University of Pittsburgh, 1970.
25) M.E. Nimni and L.A. Bavetta, *Science*, **150**, 905 (1965).