GROWTH-INHIBITION OF LEUKEMIA L-1210 ASCITES TUMOR BY COMBINATION OF CARRAGEENAN AND MITOMYCIN C *IN VIVO*

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A purified ι -carrageenan prepared by means of pronase digestion and fractional precipitation with KCl was investigated for its growth-inhibitory effect on mouse leukemia L-1210 transplanted in (C57BL/6 $\times$ DBA/2) F_1 (BDF $_1$) mice. This carrageenan specimen was not growth-inhibitory on this tumor when it was administered alone in a dose of 0.1 to 10 mg/kg/d, 4 times. However, in combination with a small amount of mitomycin C, 0.25 mg/kg/d, a remarkable tumor growth-inhibition has manifested affording a complete regression of this tumor. Acid phosphatase α -galactosidase and active oxygen generation activities of the peritoneal exudate cells in the carrageenan-administered mice were remarkably higher than those of the control mice. Therefore, the booster effect of this carrageenan preparation on antitumor activity of mitomycin C is assumed to be associated with the inflammatory activity of this sulfated polysaccharide.

Keywords—carrageenan; mitomycin C; leukemia L1210; combination therapy; antitumor activity

INTRODUCTION

Carrageenan, a strong inflammatory agent has been shown to display a variety of biological effects, *i.e.*, antilipaemic,¹⁾ anti-atherosclerotic,¹⁾ connective tissue-neoplastic,²⁾ immunosuppressive activities,³⁾ and so on. It has also been shown that carrageenan is able to activate the lysosomal enzymes of macrophages.⁴⁾ Because the latter effect can be regarded to be important in tumor cell-destroying effect evoked by macrophages, we attempted a series of tumor-growth inhibition assays of carrageenan against leukemia L-1210 ascites tumor with special reference to the lysosomal enzyme activity of macrophage obtained from the peritoneal exudate cells of mice of the same strain. The present paper reports the antitumor activity of a purified carrageenan in combination with mitomycin C and the findings on the mechanism of this activity.

MATERIALS AND METHODS

Carrageenan—A commercial product, i -car-

rageenan, Genubisco, was purchased from the Sansho Pharmaceutical Industry, Co. Ltd., Tokyo, Japan, and was further purified by digestion with pronase and by the method of Ree *et al.*^{5,6)} as follows; the carrageenan specimen, 1.0 g was dissolved in 0.05 M Tris-HCl buffer, pH 8.2, 500 ml. To the solution was added Pronase E (Kaken Chemicals Co. Ltd., Tokyo, Japan), 20 mg, and incubated at 37°C for 72 h under stirring. The solution was then heated at 100°C for 1 h. After cooling to room temperature, the solution was stirred for 30 min with equal volume 5:3 (v/v) mixture of CHCl $_3$ and n -C $_4$ H $_9$ OH. The mixture was then centrifuged at 800 $\times g$ for 20 min, and the supernatant was further treated by the same deproteinization procedure until no more aggregation of protein occurred. The clear supernatant was concentrated *in vacuo* to 200 ml, dialyzed against distilled water, 20 l, under stirring overnight, and concentrated *in vacuo* to 100 ml. This solution was poured into three volumes of abs. C $_2$ H $_5$ OH and the resultant precipitate was

collected by centrifugation, $800 \times g$ for 15 min. After washing three times with abs. C_2H_5OH 50 ml each, the precipitate was dried *in vacuo* over P_2O_5 .

The pronase-treated ι -carrageenan, 1 g, was dissolved into distilled water, 600 ml, under vigorous stirring at room temperature. To the solution was added finely powdered KCl until the concentration reached to 25 (w/v)%. The resultant gelly-like precipitate was collected by centrifugation, dehydrated with large volume of abs. C_2H_5OH , and dried *in vacuo* over P_2O_5 . The material was then dissolved in distilled water, 200 ml, and the resultant solution was dialyzed against distilled water, 20 l, for 48 h under stirring. The retentate was concentrated *in vacuo* to 100 ml, and poured into abs. C_2H_5OH , 300 ml. The resultant precipitate was collected by centrifugation, washed with abs. C_2H_5OH 3 times, each 50 ml, and dried *in vacuo* over P_2O_5 . Yield of the purified ι -carrageenan was 0.56 g or 56%, based on the amount of the crude material. The results of chemical analyses of the crude and the purified carrageenan preparations are given in Table I.

Chemical Analyses — Total carbohydrate was determined by the modified Molisch method⁷⁾ using D-galactose as the standard material. Total protein was determined by the Folin method modified by Lowry *et al.*⁸⁾ Quantitation of total sulfate was carried out by the oxygen flask combustion method by Schöniger.⁹⁾

Animal — Male mice of (C57BL/6 $\times$ DBA/2)F₁ (BDF₁) strain weighing 20 ± 2 g were used.

Tumor Cell — Mouse leukemia L-1210 cells were donated by Dr. K. Nitta of National Cancer Center Research Institute, Tokyo, Japan, and were maintained in ascites form by transplanting once a week to BDF₁ strain mouse.

Assay of Acid Phosphatase and α -Galactosidase Activities — Peritoneal exudates obtained from mice, three in one group, were centrifuged at $800 \times g$ for 5 min. Peritoneal exudates were resuspended in saline, to make a total volume of 1.0 ml (5×10^6). The incubation mixture for the acid phosphatase assay consisted of 0.1 M p -

nitrophenyl phosphate disodium salt (Wako Pure Chemical Co. Ltd., Tokyo, Japan) in 0.1 M acetate buffer pH 4.5, 0.1 ml, 0.1 M acetate pH 4.5, 0.8 ml, mouse peritoneal exudate cell suspension 0.1 ml. After incubating at 37°C for 15 min, 0.1N NaOH, 1.0 ml, was added to the mixture, and the color intensity was read at 420 nm. The incubation mixture for α -galactosidase activity had exactly the same composition excepting that 0.1 M p -nitrophenyl-D-galactopyranoside (Nakarai Chemicals Ltd., Kyoto, Japan) was used instead of p -nitrophenyl phosphate. The incubation time was 90 min, and after addition of 0.1 N NaOH, 1.0 ml, the color intensity was also read at 420 nm. Three mice/group were used, and the test was repeated 3 times.

Assay of Antitumor Activity of Carrageenan in Combination with Mitomycin C Using Mice Implanted with Leukemia L-1210 — Mice of BDF₁ strain were implanted intraperitoneally with 1×10^4 cells of L-1210 tumor cells. After 24 h tumor implantation, mice were injected intraperitoneally with 0.1 to 10 mg/kg/d of carrageenan and 1 h after the carrageenan administration, mice were injected with mitomycin C, 0.25 mg/kg/d, intraperitoneally, once a day for consecutive 4 d. The survivors after 60 d were anatomized in order to examine the presence of the ascites tumor cells. Assays were conducted using 7 or 10 mice/group and the same test was repeated 3 times.

Assay of Depressive Effect of Trypan Blue on the Antitumor Activity of Carrageenan — Two groups of BDF₁ strain mice, 7 mice each, were administered intraperitoneally with 0.4 and 0.1 ml of saline solutions of Trypan blue (10 mg/ml), 24 and 3 h before the tumor inoculation, respectively. After 24 h, these mice were implanted with 1×10^4 cells of L-1210 ascites tumor cells, and chemotherapy using carrageenan and mitomycin C was conducted by the similar manner to that described above.

Chemiluminescence Measurement — Peritoneal exudate cells, 1×10^6 , of BDF₁ mice were preincubated with 5 mM glucose and 50 μ l of luminol solution (2 mg/ml in dimethyl sulfoxide) for 10 min, then phorbol myristate acetate (PMA;

Sigma Chemical Co., St. Louis, U.S.A.) was added to the vial and chemiluminescence was measured in a Biolumat LB 9500 luminescence photometer (Berthold Co., Wildbat, West Germany) according to the method reported previously.¹⁰⁾

RESULTS

Chemical Analyses of Purified ι -Carrageenan

Table I shows the results of chemical analyses of crude and purified ι -carrageenan specimens. Sulfate content of the purified carrageenan specimen was 20%. No protein was detected in the purified ι -carrageenan.

Effect of Combination Therapy of Carrageenan and Mitomycin C against Mouse Bearing Leukemia L-1210 Ascites Tumor

BDF₁ strain mice were implanted with 1×10^4 cells of L-1210 tumor cells. After 24 h tumor implantation, mice were administered with saline solution containing 0.1 to 10 mg/kg of carrageenan and 0.25 mg/kg of mitomycin C in 0.1 ml once a day for consecutive four days. As can be

FIG. 1. Effect of Combination Treatment with Purified Carrageenan and Mitomycin C on Survival of Mice Implanted with Leukemia L-1210 Tumor Cells

● tumor bearing control, □ carrageenan 10 mg + mitomycin C 0.25 mg/kg/d $\times$ 4, ○ carrageenan 1 mg + mitomycin C 0.25 mg/kg/d $\times$ 4, △ carrageenan 0.1 mg + mitomycin C 0.25 mg/kg/d $\times$ 4, $\times$ mitomycin C 0.25 mg/kg/d $\times$ 4.

Mice of BDF₁ strain were implanted with 1×10^4 cells of L-1210 tumor cells. After 24 h tumor implantation, mice were injected intraperitoneally with carrageenan and 1 h after the carrageenan administration, mice were injected with mitomycin C intraperitoneally once a day for 4 d.

seen in Fig. 1 and Table II, about 50% of mice administered with carrageenan and mitomycin C survived more than 60 d. The results of autopsy of these mice indicated that the ascites tumor was regressed completely.

Effect of Trypan Blue on Growth-Inhibitory Activity of Carrageenan against Leukemia L-1210 Transplanted in BDF₁ Strain Mice

Table II shows the results of assay of depressive effect of a basic dye, Trypan blue, which was

FIG. 2. Kinetics of Acid Phosphatase Activity of Peritoneal Cells of BDF₁ Mice Treated with Purified Carrageenan and Mitomycin C

● control mice, ○ carrageenan 1 mg + mitomycin C 0.25 mg/kg/d $\times$ 4, △ carrageenan 1 mg/kg/d $\times$ 4, ▲ mitomycin C 0.25 mg/kg/d $\times$ 4.

FIG. 3. Kinetics of α -Galactosidase Activity of Peritoneal Cells of BDF₁ Mice Treated with Purified Carrageenan and Mitomycin C

● control mice, ○ carrageenan 1 mg + mitomycin C 0.25 mg/kg/d $\times$ 4, △ carrageenan 1 mg/kg/d $\times$ 4, ▲ mitomycin C 0.25 mg/kg/d $\times$ 4.

TABLE I. *Chemical Analyses of Carrageenans*

Material	Galactose (%)	Protein (%)	Sulfate (%)
Crude ι -carrageenan	63.0	2.2	13
Purified ι -carrageenan	69.3	0.0	20

TABLE II. *Effect of Combination Treatment with Mitomycin C and ι -Carrageenan on Survival of Mouse Implanted with L-1210 Tumor Cells*

Sample	Dose (mg/kg/d $\times$ 7)	No. of tumor-free animals at 60 d/ No. of animals tested (in different experiments)			Total No. of cures/total No. of animals tested	Percent of cures	p^a
		Exp.1	Exp.2	Exp.3			
Control		0/10	0/10	0/7	0/27	0	
ι -Carrageenan	10						
+ mitomycin C	0.25	6/10		2/7	8/17	47	$<0.01^b$
ι -Carrageenan	1						
+ mitomycin C	0.25	8/10	5/10	3/7	16/27	59	$<0.01^b$
ι -Carrageenan	0.1						
+ mitomycin C	0.25	7/10		3/7	10/17	59	$<0.01^b$
ι -Carrageenan	10	0/10		0/7	0/17	0	
ι -Carrageenan	1	0/10	1/10	0/7	1/17	6	
ι -Carrageenan	0.1	0/10		0/7	0/17	0	
Mitomycin C	0.25	5/10	1/10	0/7	6/27	22	
ι -Carrageenan	10						
+ mytomycin C	0.25			0/7	0/7	0	
+ trypan blue	0.4 + 0.1						
ι -Carrageenan	1						
+ mytomycin C	0.25			0/7	0/7	0	
+ trypan blue	0.4 + 0.1						
ι -Carrageenan	0.1						
+ mytomycin C	0.25			0/7	0/7	0	
+ trypan blue	0.4 + 0.1						

a) The test criterion is χ^2 . b) Versus the group treated with mitomycin C alone.

shown to inhibit the macrophage function,¹¹⁾ on antitumor activity of carrageenan and mitomycin C. It is obvious that the group of mice treated with ι -Carrageenan mitomycin C, and Trypan blue showed a nearly identical survival time as those treated with Trypan blue and non-treated control, suggesting that this antitumor activity might depend on the peritoneal macrophages.

Enzyme Activities of Peritoneal Exudate Cells

Obtained from the Mice Treated with Carrageenan and Mitomycin C

Figures 2 and 3 show the results of kinetic study on the enhancing effect of combination therapy of ι -carrageenan and mitomycin C on acid phosphatase and α -galactosidase activities of mouse peritoneal exudate cells. It is obvious that the induced activities of both acid phosphatase and α -galactosidase are stronger than those

induced by the administration of carrageenan alone.

Chemiluminescence Response by Peritoneal Exudate Cells in Mice Treated with Carrageenan and Mitomycin C

The results of chemiluminescence response by peritoneal exudate cells from the mice administered with carrageenan and mitomycin C is shown in Fig. 4. Peritoneal exudate cells administered with carrageenan and mitomycin C showed a strong chemiluminescence response as compared with those of peritoneal exudate cells from the control mice. Whereas, response by the peritoneal exudate cells of mice administered with carrageenan or with mitomycin C alone was very weak and no convincing difference with that of the peritoneal exudate cells obtained from normal control mice was evident.

DISCUSSION

Various polysaccharides have been shown to display growth-inhibitory effect against mouse-implanted tumors, *i.e.*, mannan from bakers' yeast,^{12,13)} Schizophyllan from *Schizophyllum*

*commune*¹⁴⁾ and Lentinan from edible mushroom, *Lentinus edodes*¹⁵⁾ and so on. These polysaccharides were shown to be capable of exerting adjuvant action enhancing both humoral and cellular antibody responses to the host animal.¹⁶⁻¹⁹⁾ Additionally, acidic polysaccharides such as dextran phosphate,^{20,21)} and mannan phosphate,^{22,23)} acidic mannan fraction,²⁴⁾ and chondroitin sulfate A²⁵⁾ were also shown the activity. In the present study, we studied antitumor activities of a sulfated polysaccharide, *v*-carrageenan, a constituent of seaweed, *Eucheuma spinosum*, known to be a potent inflammatory agent.^{26,27)} The results of the present paper demonstrate that carrageenan is able to exhibit an antitumor effect against leukemia L-1210 in combination with mitomycin C. Because carrageenan possesses the enhancing effect of lysosomal enzymes of macrophages as pointed out by Schorlemmer and Allison *et al.*,^{28,29)} the antitumor activity of this sulfated polysaccharide is also assumed to elevate the tumor-cell injuring activity of macrophages. It was also reported that some immunoadjuvants were able to enhance the release the superoxide anion from macrophage.³⁰⁾ On the other hand, Nathan *et al.* showed the results that H_2O_2 , released from macrophages and leukocytes have an antitumor activity.³¹⁾ In combination therapy with carrageenan and mitomycin C, elevation of both acid phosphatase and α -galactosidase activities was observed on peritoneal exudate cells of normal mice, and the release of superoxide anion was also evident under the same conditions. These results indicate that the administration of carrageenan and mitomycin C elicits the activation on some cell population(s) in peritoneal exudate cells of mice. In order to explain whether this antitumor activity was related to the function of macrophage, an inhibition study using Trypan blue was conducted. Because the vanishment of antitumor activity of carrageenan and mitomycin C by treatment with this dye was evident, a prove for the participation of macrophage in this antitumor activity was obtained. On the other hand, carrageenan is known to be one of the macrophage function

FIG. 4. Chemiluminescence Response by Peritoneal Cells in BDF₁ Mice Administered with Carrageenan and Mitomycin C

—○— carrageenan 1 mg + mitomycin C 0.25 mg/kg/d × 4, — control mice, — — — SD.

inhibitors when it was administered in a large dose.³²⁾ However, the administration of carrageenan in such a small dose as used in the present study induced a significant enhancement of peritoneal exudate cells. Because the administration of carrageenan alone did not evoke biological effects including antitumor activity, enhancement of lysosomal enzymes, and the release of superoxide anion, it appears conceivable to state that this antitumor activity is partially responsible for the synergistic effect by mitomycin C. This combination effect seems to be closely similar to those observed in our previous studies,^{20-23,25)} using several acidic polysaccharides, *O*-palmitoyldextran phosphate, yeast mannan derivatives, and chondroitin sulfate A, with mitomycin C.

Basing on the above findings, it will be reasonable to conclude that the mechanism of growth-inhibitory effect of combination therapy with ι -carrageenan and mitomycin C on mice bearing leukemia L-1210 ascites tumor involves enhancement of peritoneal exudate cells, especially peritoneal macrophages to elevate lysosomal enzyme levels and to release large amounts of active oxygens.

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