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Antitumor Activity of Carboxymethylglucans Obtained by Carboxymethylation of (1→3)-β-D-Glucan from *Alcaligenes faecalis* var. *myxogenes* IFO 13140*

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Abstract—*Soluble carboxymethylglucans were obtained by carboxymethylation of insoluble* (1→3)-β-D-*glucan from* Alcaligenes faecalis *var.* myxogenes *IFO* 13140. *Carboxymethylglucan with* $\overline{DS}$ 0.47 ($\overline{DS}$, *number-average degree of carboxymethyl groups substituted per anhydroglucose unit) strongly inhibited growth of sarcoma* 180 *solid-type tumor when injected daily i.p.* (3–40 mg/kg) *or i.v.* (5 mg/kg) *for* 10 days. *It also strongly inhibited growth of Sarcoma* 180 *when injected i.p. in a single dose of* 20–200 mg/kg 7 days *after tumor transplantation.*

The $\overline{DS}$ *of carboxymethylglucans was found to be closely correlated with their antitumor activity, compounds with a* $\overline{DS}$ *of about* 1.0 *per glucose residue having less activity.*

This chemically modified glucan seemed to have a host-mediated action, because it had no cytotoxic effect in vitro *and because it was effective when injected before tumor transplantation.*

INTRODUCTION

PREVIOUSLY we found that a non-toxic (1→3)-β-D-glucan from *Alcaligenes faecalis* var. *myxogenes* (IFO 13140) strongly inhibited growth of Sarcoma 180, solid-type tumor [1]. It was not cytocidal to tumor cells in culture, but had strong antitumor activity *in vivo* when injected at a daily dose of more than 5 mg/kg for 10 days. When given in this way, it was as effective before tumor inoculation as after tumor inoculation. Although this glucan has strong antitumor activity, its potential value is limited by its low solubility in water. We, therefore, attempted to obtain a water-soluble derivative that retained the antitumor activity of the parent glucan. Many polysaccharides of different origins have recently been reported [2], but there have been few attempts to modify them chemically. We attempted to increase the solubility by introduction of hydrophilic groups and found that carboxymethylation to the glucan increased the solubility and antitumor potency without resulting in any detectable toxicity to the host animals.

This paper reports the strong antitumor activity of the water-soluble carboxymethyl (1→3)-β-D-glucan. The correlation between the activity and the extent of carboxymethylation is also discussed.

MATERIALS AND METHODS

1. *Animals and tumor*

Female ICR-CRJ mice, weighing about 24 g, were purchased from Charles River Co. Inc., Japan. Sarcoma 180, initially obtained from the Sloan–Kettering Institute, New York, N.Y., has been maintained in our Institute in an ascites form. Unless otherwise stated, 0.05 ml (6×10^6 cells) of the 7-day-old ascites tumor was transplanted s.c. into the right groin of mice. We have never observed spontaneous regression of this tumor.

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2. *Assay of antitumor activity*

Test samples at a suitable concentration in distilled water, were sterilized for 20 min at 120°C and then injected into mice daily (0.1 ml, i.p.), starting 24 hr after tumor implantation, unless otherwise noted. Mice were kept under observation for 5 weeks and then killed for final evaluation of the effect of treatment on tumor growth. Inhibition ratios were calculated by the following formula:

$$\text{Inhibition ratio } (\%) = (A-B)/A \times 100,$$

where A is the average tumor weight of the control group and B is that of the treated group.

3. In vitro *culture of Sarcoma* 180 *cells*

The ascites tumor cells (10^5) of Sarcoma 180 were incubated in a CO_2 gas incubator at 37°C for 24 hr in 5 ml of medium containing various concentrations of carboxymethylglucan. Then their viability, estimated by staining with 0.17% trypan blue, was compared with that of control cells incubated in the same medium without the glucan.

Eagle's minimum essential medium with 20% calf serum was used for suspension cultures.

4. *Carboxymethylation*

The content of carboxymethyl groups in carboxymethylglucan depends on the conditions used for carboxymethylation. The degree of substitution (DS, the average number of carboxymethyl groups substituted per anhydroglucose unit) was determined by the method of Eyler *et al.* [3]. (1→3)-β-D-Glucan with a number-average degree of polymerization of 540 from the culture filtrate of *Alcaligenes faecalis* var. *myxogenes* IFO 13140 [4] was obtained from Takeda Chemical Industries Ltd., Osaka, Japan. This glucan was carboxymethylated in the following two ways:

(A) A suspension of 3 g of the glucan in 80 ml of isopropyl alcohol was stirred at room temperature for 30 min. Then, 8 ml of a 30% solution of sodium hydroxide was slowly added with stirring over a period of about 60 min. Vigorous stirring was continued at room temperature for about 90 min to prevent gel formation. Then, 3.6 g of monochloroacetic acid was added and the mixture was stirred at 60°–70°C for 5 hr. The product was recovered by filtration and thoroughly washed with a mixture of methanol and acetic acid (7:3, v/v). The precipitate was collected by filtration, washed successively with 80% aqueous methanol, methanol and acetone and dried under reduced pressure. This procedure yielded 2.9 g of carboxymethylglucan (DS; 0.47 or 0.65).

(B) A suspension of 1.5 g of the glucan in 40 ml of isopropyl alcohol was stirred at room temperature for 30 min. Then, 4 ml of a 30% solution of sodium hydroxide was added in 1 ml portions at 15-min intervals and stirring was continued at room temperature for 90 min. Then 1.8 g of monochloroacetic acid was added in 3 portions at intervals of 10 min, and the mixture was stirred at 50°C for 150 min. The product was collected by centrifugation, dissolved in 40 ml of water and neutralized with acetic acid. The neutral solution was mixed with 90 ml of methanol and the resulting precipitate was collected by centrifugation. It was washed well with a mixture of 200 ml of 80% aqueous methanol and 100 ml of ethanol and, then, with a mixture of 200 ml of 80% aqueous ethanol and 200 ml of ether and then lyophilized, to give 2.0 g of carboxymethylglucan (DS; 0.98 or 1.03).

RESULTS

1. *Effects of various doses of carboxymethyl* (1→3)-β-D-*glucan of* DS 0.47 *on Sarcoma* 180 *solid-type tumor in ICR mice*

The effect of carboxymethylglucan was tested on Sarcoma 180 solid-type tumor transplanted s.c. into mice. As shown in Table 1, the carboxymethylglucan had a strong antitumor effect when given in daily doses of 3–40 mg/kg i.p. for 10 days from the day after tumor transplantation. There was no detectable toxicity to mice with these doses, the LD_{50} being over 2500 mg/kg i.p. in mice.

A single dose of 20–200 mg/kg i.p. at 7 days after s.c. transplantation of Sarcoma 180 also caused complete tumor regression. These doses resulted in tumor inhibition ratios of over 88.5%, and caused regression and complete disappearance of the tumors in many animals.

Smaller or larger doses of 10 and 400 mg/kg had less antitumor activity, which resulted in tumor inhibition ratios of 42 and 79%, respectively, and did not cause tumor regression. Daily injections of 10 mg/kg for 10 days had a similar effect to a single injection of 100 mg/kg at 7 days after tumor implantation. Namely, the implanted tumor grew to about the same extent as in control mice for 2 weeks, and then regressed, disappearing almost completely within 5 weeks.

Table 1. *Effect of carboxymethyl* (1→3)-β-D-*glucan with* DS 0.47 *on Sarcoma* 180 *solid form in mice*

Dose (mg/kg × days)	Days of sample injection	Average tumor weight (g)	Tumor inhibition ratio (%)	Complete regression*
1 × 10	+1 to +11	0.77±0.91†	78.9	1/6
3 × 10	+1 to +11	0.03±0.08	99.2	5/6
Control		3,65±1.38		0/6
5 × 10‡	+1 to +11	0.84±1.88	88.1	4/5
5 × 10§	+1 to +11	3.77±1.45	46.4	0/5
Control		7.03±2.88		0/10
10 × 10	+1 to +11	0.07±0.16	96.8	5/6
20 × 10	+1 to +11	0.21±0.28	90.3	3/6
40 × 10	+1 to +11	0.18±0.10	91.7	1/6
Control		2.17±1.09		0/6
10 × 1	+ 7	3.44±2.54	41.6	0/5
20 × 1	+ 7	0.68±0.94	88.5	2/6
60 × 1	+ 7	0	100	6/6
100 × 1	+ 7	0.03±0.06	99.5	4/6
200 × 1	+ 7	0.08±0.15	98.7	4/6
400 × 1	+ 7	1.23±1.19	79.1	0/6
Control		5.89±3.44		0/6
100 × 1	− 7	1.98±2.16	70.4	1/6
100 × 1	− 3	0.55±0.52	91.8	2/5
100 × 1	+ 4	0.05±0.10	99.3	5/6
100 × 1	+10	0.23±0.28	96.6	4/6
100 × 1	+14	0.42±0.60	93.7	2/6
100 × 1	+21	1.74±2.06	74.0	1/5
Control		6.68±1.72		0/6

All mice were inoculated s.c. with tumor on day 0.
*No. of tumor free mice/No. of mice tested.
†Values are means ± standard deviations.
‡Administered i.v.
§Administered s.c.

In contrast, carboxymethylglucan did not inhibit the growth of ascitic Sarcoma 180 or prolong the life of mice with ascites tumors. The average survival time (12.7 days) of mice inoculated i.p. with 6×10^6 cells of Sarcoma 180 ascites tumor and treated i.p. with 10 mg/kg daily for 5 days of carboxymethylglucan (DS, 0.47) from the day after transplantation did not differ appreciably from that (12.1 days) of the control group.

2. *Effects of single injections of carboxymethyl* (1→3)-β-D-*glucan with* DS 0.47 *on various days before or after s.c. transplantation of Sarcoma* 180

The effectiveness of pretreatment of animals is characteristic of the antitumor activity of polysaccharides. Table 1 shows that carboxymethylglucan also showed antitumor activity when administered before tumor transplantation. When injected as a single dose of 100 mg/kg, it had a marked effect (inhibition ratio, 91.8%) 3 days before s.c. transplantation of Sarcoma 180, but less effect 7 days before tumor transplantation (inhibition ratio, 70.4%). The same dose 4, 10 or 14 days after tumor transplantation resulted in an inhibition ratio of over 93%, with regression and complete disappearance of tumors in many animals. Even a single injection 21 days after tumor transplantation, resulted in a significant inhibition ratio of 74% (Table 1).

3. *Effects of carboxymethyl* (1→3)-β-D-*glucan with* DS 0.47 *by various routes of administration*

Carboxymethylglucan was administered at a dose of 5 mg/kg i.v. or s.c. for 10 consecutive days from 24 hr after s.c. inoculation of Sarcoma 180 cells (Table 1). It showed high activity when given i.v. (inhibition ratio, 88%) and less activity when given s.c. (inhibition ratio, 46%). It had no appreciable effect when given orally, even at a high dose of 1700 mg/kg.

4. *Effects of carboxymethyl* (1→3)-β-D-*glucans with various contents of carboxymethyl groups*

The effect of the content of carboxymethyl groups on activity was investigated using 4 samples of carboxymethylglucan with different

contents of carboxymethyl groups. The results of bioassay are shown in Table 2. When given at a dose of 10 mg/kg i.p. daily for 10 days, carboxymethylglucan with a content of carboxymethyl groups of 0.65 or less per glucose residue had strong antitumor activity, causing complete regression of tumors in many animals, whereas preparations with contents of carboxymethyl groups of 0.98 or more per glucose residue had lower antitumor activities. effective when given as a single injection i.p. 0.47 resulted in a tumor inhibition ratio of almost 100% and a high rate of complete tumor regression; moreover, even a dose of 1 mg/kg, resulted in a significant inhibition ratio (78.9%). Similar doses of the parent glucan have little effect. The antitumor potencies of these carboxymethylglucans are greater than that reported for carboxymethylpachymaran [5]. Carboxymethylglucan was even

Table 2. Effects of carboxymethyl (1→3)-β-D-glucans with various contents of carboxymethyl groups at a dose of 10 mg/kg for 10 days on Sarcoma 180 solid form

$\overline{DS}$ of carboxymethyl group	$[\alpha]_D^{25}$ in H_2O	Average tumor weight (g)	Tumor inhibition ratio (%)	Complete regression*
0.47	+6.0° (c=0.77)†	0	100	6/6
0.65	−2.6° (c=1.64)	0.11±0.17‡	97.1	4/6
0.98	−2.0° (c=1.53)	1.33±2.14	64.4	3/6
1.03	−2.3° (c=1.58)	1.34±2.45	64.2	2/5
Control		3.74±1.44		0/6
0		0.01±0.02	99.8	5/6
Control		6.00±2.36		0/6

*No. of tumor-free mice/No. of mice tested.
†Measured in 0.1N NaOH.
‡Values are means ± standard deviations.

DISCUSSION

Previously, we found that (1→3)-β-D-glucan (from *Alcaligenes faecalis* var. *myxogenes* IFO 13140) has antitumor activity, but is very insoluble in water, so that it could not be administered satisfactorily i.v., the most suitable route from practical purposes. Moreover, when autoclaved, it forms a very elastic resilient gel that does not break, as agar gel does, when pressed between the fingers. In the present work we found that on carboxymethylation (1→3)-β-D-glucan becomes water soluble, without loss of its antitumor activity. Carboxymethylglucan with $\overline{DS}$ 0.47 was soluble in distilled water (70 mg/ml), and the clear solution could be sterilized before experiments by autoclaving.

Analysis of the ^{13}C nuclear magnetic resonance spectra of the carboxymethylglucan in deuterium oxide suggested that the carboxymethylation occurs principally at C-6 (Saitó and Sasaki, unpublished data).

The carboxymethylglucans with $\overline{DS}$'s of 0.47 and 0.65 used in this work showed strong antitumor activity at lower doses than the effective dose of the parent glucan [1]. A dose of 3 mg/kg of carboxymethylglucan of $\overline{DS}$ after the tumor had become completely established (14 days after transplantation) or when injected i.v. at a dose of 5 mg/kg. The effective dose-range (20–200 mg/kg) of carboxymethylglucan was wider than that (60–100 mg/kg) of the parent glucan. This is especially useful for therapeutic purposes.

No information is available on whether carboxymethylglucan has a direct antitumor effect, or whether it is effective after hydrolysis to glucan. Probably carboxymethylglucan is converted to glucan *in vivo*, but carboxymethyglucan may have a direct effect as a polyanion due to its carboxylic acid.

The antitumor actions of both carboxymethylglucan and the parent glucan, are probably host-mediated, because neither compound has any direct cytocidal effect on tumor cells *in vitro* (data not shown) and both compounds are effective when injected before tumor transplantation.

Our results showed that the degree of substitution of carboxymethyl groups affected the antitumor activity: activity was lower when the degree of substitution with carboxymethyl groups was high. For antitumor activity, the limit to the content of this group seems to be about 1.0 per glucose residue. It is interesting

in this connection that deacetylation of GE-3, a polysaccharide from *Gyrophera esculenta* containing 4–10% of acetyl groups, lowers the antitumor activity, and that complete acetylation of GE-3 yields inactive products [6]. These findings suggest that the hydroxyl group of polysaccharides may be involved in interaction with some substance in the animal body necessary for biological activity and may also participate in maintenance of some essential conformational structure [7–9]. Extensive substitution with carboxymethyl groups may increase the solubility in water, but also alter the molecular orientation, the lateral order and the conformational structure, eventually causing loss of antitumor activity.

The present study partially clarifies the relationship between chemical modification and growth inhibitory activity of glucan. Further studies are in progress on the mode of antitumor action using ^{14}C-labelled carboxymethylglucan.

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