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Dependence on Chain Length of Antitumor Activity of (1 → 3)-β-D-Glucan from *Alcaligenes faecalis* var. *myxogenes*, IFO 13140, and Its Acid-degraded Products[1]

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ABSTRACT

The well-defined (1 → 3)-β-D-glucan with ($\overline{DP}_n$) 540, produced by cultivation of *Alcaligenes faecalis* var. *myxogenes* (IFO 13140), a mutant of a soil bacterium, had marked inhibitory activity against the s.c.-implanted Sarcoma 180 at 5 to 50 mg/kg for 10 days. It also exhibited very high activity in doses of 60 and 100 mg/kg i.p. by a single injection at 7 days after the initial s.c. transplantation of Sarcoma 180. The inhibition ratios observed with Ehrlich carcinoma, NTF (Nakahara-Tokuzen-Fukuoka) reticulum cell sarcoma, and CCM adenocarcinoma were somewhat less but were still significant. On the other hand the treatment failed to inhibit the growth of ascites Sarcoma 180 or to induce prolongation of life span. The mechanism of action of this glucan was considered to be host mediated because of a lack of effect *in vitro* and also because of the effectiveness of pretreatment of animals by injection before transplantation of a tumor. The results from the bioassay study of the lower-molecular-weight (1 → 3)-β-D-glucans prepared from the glucan with number-average degrees of polymerization ($\overline{DP}_n$) 540 by hydrolysis with formic acid or sulfuric acid showed that the glucans with $\overline{DP}_n$ > 50 inhibited the growth of Sarcoma 180 implanted s.c. in mice, whereas the result of the schedule-dependent effect was obtained in the pretreatment of animals by the glucan with $\overline{DP}_n$ 50. By i.v. administration, even the glucan with $\overline{DP}_n$ 16 showed a strong antitumor effect comparable to that of the glucan with $\overline{DP}_n$ 540.

INTRODUCTION

Polysaccharides with antitumor activity against certain allogeneic tumors, particularly Sarcoma 180 in mice, have been isolated from diverse sources including higher plants, fungi, lichen, bacteria, and yeasts (23). The nature of their antitumor action is not entirely clear, but the polysaccharides from botanical sources cannot be shown to exert any direct action on tumor cells. Their antitumor action must therefore be considered to be dependent on the reaction of the host; *i.e.*, their effect is host mediated (9). The structure and chemical purity of many of the polysaccharides thus far reported have not been completely confirmed, and the attempt to correlate activity with structure is difficult. Therefore, 1 of the major areas of research effort is the search for new polysaccharides that have simple structure and high homogeneity. Such studies should lead to the discovery of more effective antitumor polysaccharides from considerations of the relationship between chemical structure and biological activity.

Recently, a thermally gelable (1 → 3)-β-D-glucan, curdlan-type polysaccharide that consists entirely of β-D-(1 → 3)-linked D-glucose residues and contains no other linkages, as in pachyman and laminarian, was obtained by cultivation of *Alcaligenes faecalis* var. *myxogenes* (IFO 13140), a mutant of a soil bacterium (5, 11, 14). It seems most appropriate to use this polysaccharide, which is free from ambiguity due to other linkages, for a study of the structure-activity relationship in the antitumor activity of (1 → 3)-β-D-glucan. The studies reported here examine the antitumor effect of this polysaccharide and its acid hydrolysates against Sarcoma 180 and other transplantable solid tumors in mice.

MATERIALS AND METHODS

Mice. Experiments were carried out on female ICR-JCL mice, weighing about 23 g, purchased from CLEA Japan Inc.

Tumors. Sarcoma 180 and Ehrlich carcinoma were initially supplied by Sloan-Kettering Institute, New York, N. Y., and have been maintained in our Institute in an ascites form. NTF (Nakahara-Tokuzen-Fukuoka) reticulum cell sarcoma was first reported as "Friend virus-associated sarcoma" by Nakahara *et al.* (10). It has since become, spontaneously, Friend virus free (hence the change of designation) and has been maintained in this Institute in ICR-JCL mice by serial i.p. passages (10, 21).

CCM adenocarcinoma is a spontaneous mammary adenocarcinoma that originated and has been maintained for several years in random-bred Swiss mice by serial s.c. transplantations of solid grafts, always with 100% takes and with very little fluctuation in the rate of growth (3). The ascites form of this tumor was produced 1 year ago by an artificial means of cell dispersal and trypsin treatment (20). This ascites variant has also been maintained in ICR-JCL mice by serial i.p. passages, showing a uniform survival period for the mice.

Unless otherwise stated, 0.05 ml (about 6×10^6 cells) s.c. of the 7-day-old ascites tumors mentioned previously was transplanted into the right groin of mice. We have never observed any spontaneous regression of these tumors.

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Assay of Antitumor Activity. The test samples suspended in distilled water in adequate concentrations (injection volume, 0.1 ml i.p.) were injected daily, starting 24 hr after tumor implantation unless otherwise noted. All mice were kept under observation for 5 weeks and then were sacrificed for the final evaluation of the effect of treatment on tumor growth. The inhibition ratios were calculated by use of 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. Complete regression indicates the ratio of the number of mice showing complete regression to the number of mice tested.

(1 → 3)-β-D-Glucans. The purified (1 → 3)-β-D-glucan [number-average degrees of polymerization ($\overline{DP}_n$) 540], which is obtained from the culture filtrate of *A. faecalis* var. *myxogenes* (IFO 13140), was supplied by Takeda Chemical Industries Ltd., Osaka, Japan. This glucan was insoluble in water and in most organic solvents, but it was soluble in alkaline solution and dimethyl sulfoxide and was usually soluble in solutions containing hydrogen bond-breaking reagents. The IR spectrum showed a band at 890 cm^{-1}, indicating the presence of the β-glycosidic bond. No significant absorption in the UV spectrum was observed. This glucan showed a positive reaction to Molisch's reagent, phenolsulfuric acid test and anthrone reagent. The pH of its aqueous suspension was between 6.0 and 7.0. Its specific rotation was $[\alpha]_D^{22}$ − 17 (*c*, 1.0 in dimethyl sulfoxide), $[\alpha]_D^{22}$ +31 ± 6° (*c*, 1.0 in 0.1 N NaOH), and $[\alpha]_D^{25}$ = +22° (*c*, 2.0 in HCOOH), respectively. Elementary analysis of this glucan showed C, 40.13%; H, 6.74%; ash, 3.9%. No nitrogen, phosphorus, and sulfur were detected.

Lower-molecular-weight glucans ($\overline{DP}_n$'s 7, 16, 24, 39, 50, 68, 82, and 125) were prepared from (1 → 3)-β-D-glucan ($\overline{DP}_n$ 540) by hydrolysis with 90% formic acid (95°; 40 min) or 4 N sulfuric acid (60°; 30 to 120 min) according to the methods described by Ogawa *et al.* (12) and Sasaki *et al.* (18). General properties of these lower-molecular-weight glucans were similar to that of the native glucan ($\overline{DP}_n$ 540), and gel filtration patterns of these preparations on a Sephadex G-200 column gave each symmetrical peak, which indicated that they had a normal distribution with regard to the degree of polymerization. The glucan with $\overline{DP}_n$ 7 was soluble, and the glucans with $\overline{DP}_n$'s 16, 24, 39, and 50 were slightly soluble in water, whereas those with $\overline{DP}_n$'s 68, 82, and 125 were insoluble. The $\overline{DP}_n$'s of the respective glucans were determined by the method of Manners *et al.* (8).

***In Vitro* Culture of Sarcoma 180 Cells.** The ascites tumor cells (5 × 10^6 cells) of Sarcoma 180 were incubated in a CO_2 incubator at 37° in a medium containing the glucan in various concentrations. After a 24-hr incubation the cells were stained with 0.17% trypan blue to estimate their viability, which was compared with that of control cells incubated in the same medium without the glucan. Eagle's minimum essential medium with 20% calf serum added was used for the suspension cultures.

RESULTS

Effect of Various Doses of (1 → 3)-β-D-Glucan with $\overline{DP}_n$ 540 against Sarcoma 180 Solid-type Tumor in ICR-JCL Mice. As shown in Table 1, (1 → 3)-β-D-glucan with $\overline{DP}_n$ 540 showed a pronounced antitumor effect against Sarcoma 180 solid tumor, which was transplanted s.c. in ICR-JCL mice, with doses of 5 to 50 mg/kg i.p. given once a day for

Table 1
Effect of (1 → 3)-β-D-glucan with $\overline{DP}_n$ 540 against Sarcoma 180 in mice

Sample	Dose (mg/kg × days)	Tumor wt (g)	% Tumor inhibition ratio	Complete regression	Days of sample injections[a]	Survival (days)[b]
		Sarcoma 180 solid tumor				
D-Glucan	1 × 10	4.80 ± 2.16[c]	20.0	0/6		
	3 × 10	3.58 ± 1.80	40.3	1/6		
	5 × 10	0.02 ± 0.05	99.7	5/6		
	7 × 10	0.73 ± 1.79	87.8	5/6		
	10 × 10	0.01 ± 0.02	99.8	5/6		
	15 × 10	0.10 ± 0.22	98.3	4/5		
	20 × 10	0	100	5/5		
	25 × 10	0.42 ± 0.69	93.0	3/6		
	50 × 10	1.19 ± 1.60	80.2	1/6		
Control		6.00 ± 2.36		0/6		
Autoclave-treated D-glucan	10 × 10	0.29 ± 0.71	92.1	5/6		
Control		3.67 ± 2.87		0/6		
		Sarcoma 180 ascites tumor				
D-Glucan	10 × 5				−5 to −1	14.7 ± 3.56[c]
	10 × 10				−5 to +5	13.8 ± 2.39
Control						11.6 ± 2.30

[a] Sarcoma 180 cells were transplanted on Day 0.
[b] Average of ten mice/test dose.
[c] Average ± S.D.

10 days, from the day after transplantation. There was no detectable toxicity with the dose that kills 50% of the animals > 2500 mg/kg i.p. after injection in mice.

In the antitumor action of polysaccharides, there is an interesting phenomenon of an optimal dose, which differs from the action of cytotoxic agents in general. The glucan gave complete regression of Sarcoma 180 transplanted in ICR-JCL mice in doses of 60 and 100 mg/kg i.p. by a single injection at 7 days after the initial s.c. transplantation of the tumor. The tumor inhibition ratio was almost 100% with a high rate of complete tumor regression. However, in smaller or larger doses (20, 200, and 400 mg/kg), this glucan showed a decrease in its antitumor activity. Its tumor inhibition ratio was markedly reduced to 30, 52, and 20%, respectively, and regression of tumors was not observed. The dose-response curve was characteristic, and a plot of the data showed optimal doses between 60 and 100 mg/kg (Chart 1).

The rate of tumor growth in mice given successive injections for 10 days of 10 mg/kg was the same as that in mice treated with 100 mg/kg by a single injection at 7 days. In these experiments the implanted tumor grew for up to 2 weeks to approximately the same extent as it did in control mice, began gradually to regress in the treated group, and disappeared almost completely by 5 weeks. No recurrence of the tumor was observed even when the animals were left untreated.

Effect of Autoclave-treated (1 → 3)-β-D-Glucan with $\overline{DP}_n$ 540 against Sarcoma-180 in ICR-JCL Mice. The gel of (1 → 3)-β-D-glucan, obtained by heating a 2% suspension at 90°, is very elastic and resilient and does not break, as agar gel does, when it is pressed between the fingers. When the glucan with $\overline{DP}_n$ 540 was autoclaved, no loss of its activity was observed (Table 1). The gel-forming glucan suspension, which was powdered after autoclave treatment (120°; 25 min; 1 kg/sq cm), was still effective against Sarcoma 180 at a dose of 10 mg/kg for 10 days; it showed a 92.1% inhibition ratio with complete tumor regression in 5 of 6 mice tested. This result indicates that the sample can be sterilized by autoclaving without loss of its activity.

Effect of (1 → 3)-β-D-Glucan with $\overline{DP}_n$ 540 against Sarcoma 180 Ascites Tumor in ICR-JCL Mice. Table 1 also shows lack of antitumor activity of the glucan with $\overline{DP}_n$ 540 against Sarcoma 180 ascites tumor transplanted i.p. in ICR-JCL mice. The average survival time of mice given 7-day-old Sarcoma 180 ascites tumor by transplantation (6×10^6 cells) i.p. and 10 mg glucan per kg i.p. by pretreatment for 5 days or by combined pretreatment and posttreatment for 10 days were 14.7 and 13.8 days, respectively. This did not differ appreciably from the survival time of 11.6 days in the control group.

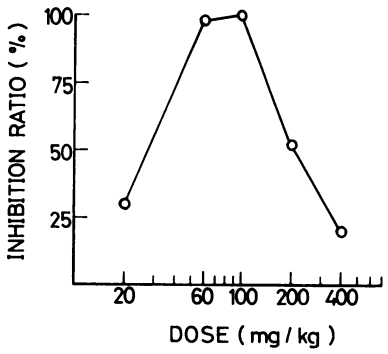

Chart 1. Dose-response curve for the antitumor activity of (1 → 3)-β-D-glucan with $\overline{DP}_n$ 540 by a single i.p. injection at 7 days after the initial s.c. transplantation of Sarcoma 180. Ten mice were used for each test dose.

Effect of (1 → 3)-β-D-Glucans with Various Chain Lengths. The study of the minimal molecular weight required for the antitumor activity of polysaccharide was investigated by use of 8 (1 → 3)-β-D-glucans of different molecular weight, obtained from D-glucan ($\overline{DP}_n$ 540) by hydrolysis with formic acid or sulfuric acid. The results of bioassay are shown in Table 2. Glucans with $\overline{DP}_n > 50$ indicated a strong antitumor activity with many complete regressions against Sarcoma 180 by consecutive injections at a dose of 10 mg/kg i.p. for 10 days, whereas the glucans with $\overline{DP}_n < 39$ showed a slight effect.

Effect of Pretreatment with (1 → 3)-β-D-Glucans of Various Chain Lengths. The effectiveness of pretreatment of animals is characteristic of the antitumor activity of polysaccharides. Table 3 shows the antitumor effect of various chain lengths of glucans administered before tumor transplantation. Glucans with $\overline{DP}_n > 50$ were also found to inhibit the growth of Sarcoma 180 when they were given 1/day for

Table 2

Effect of linear (1 → 3)-β-D-glucans of various chain lengths against Sarcoma 180 solid-form tumor at a dose of 10 mg/kg for 10 days

$\overline{DP}_n$ of D-glucan	Tumor wt (g)	% tumor inhibition ratio	Complete regression
7	2.39 ± 3.07[a]	56.0	0/6
16	1.54 ± 1.57	71.9	1/6
24	3.60 ± 2.37	34.3	0/6
39	2.03 ± 1.29	62.9	1/6
50	0	100	6/6
68	0.03 ± 0.06	99.5	5/6
82	0	100	6/6
125	0	100	6/6
540	0.20 ± 0.29	96.3	3/5
Control	5.46 ± 3.08		0/6

[a] Average ± S.D.

Table 3

Effect of pretreatment with (1 → 3)-β-D-glucans of various chain lengths against Sarcoma 180 solid-form tumor at a dose of 20 mg/kg for 5 days

$\overline{DP}_n$ of D-glucan	Days of sample injections[a]	Tumor wt (g)	% tumor inhibition ratio	Complete regression
39	−5 to −1	5.52 ± 2.71[a]	45.3	0/6
50	−5 to −1	1.30 ± 2.80	87.1	3/5
68	−5 to −1	0.35 ± 0.86	96.5	5/6
Control		10.10 ± 2.82		0/6
540	−5 to −1	0.37 ± 0.85	92.7	4/6
Control		5.06 ± 1.39		0/5
39	−7 to −3	8.48 ± 2.89	−13.8	0/5
50	−7 to −3	7.98 ± 4.95	−7.1	0/5
68	−7 to −3	0.75 ± 1.68	89.9	4/5
540	−7 to −3	0.18 ± 0.37	97.6	3/5
Control		7.45 ± 2.02		0/5

[a] Sarcoma 180 cells were transplanted on Day 0.
[b] Average ± S.D.

5 days at a dose of 20 mg/kg and tumor cells were transplanted 24 hr after the last injection. When tumor cells were transplanted 3 days after the last injection, however, the glucan ($\overline{DP}_n$ 50) did not show its antitumor activity; the inhibition ratio was −7.1%.

Effect of (1 → 3)-β-D-Glucan with $\overline{DP}_n$ 540 against Various Transplantable Tumors in Mice. From 24 hr after s.c. inoculation of the transplantable tumors, various doses of this glucan ($\overline{DP}_n$ 540) were consecutively administered for 10 days. The glucan was effective against Ehrlich carcinoma solid-type tumor at a dose of 20 mg/kg; it showed an inhibition ratio of 85.9%, with complete tumor regression in 2 of 6 mice tested. On the other hand this glucan gave a slight effect with no complete tumor regression at doses of 10 and 40 mg/kg (inhibition ratios, 33.1 and 73.7%, respectively). This glucan was also found to inhibit the growth of NTF reticulum cell sarcoma and CCM adenocarcinoma; the inhibition ratio was 95.0% against the NTF sarcoma at a dose of 10 mg/kg and was 76.9° against the CCM adenocarcinoma at a dose of 20 mg/kg. Complete regression of both tumors occurred in 2 of the 6 mice tested.

Comparison of the Effect of the Glucans ($\overline{DP}_n$ 16 and 540) Administered by Various Routes of Injection. From 24 hr after s.c. inoculation of Sarcoma 180 cells into ICR-JCL mice, 5 mg/kg of (1 → 3)-β-D-glucans ($\overline{DP}_n$'s 16 and 540) were administered by various routes for 10 days. Although i.p. or s.c. administration of the glucan with $\overline{DP}_n$ 16 was slightly effective (inhibition ratios, 68 and 63%, respectively), this low-molecular-weight glucan was highly effective by i.v. administration; the inhibition ratio was 85% with complete tumor regression in 4 of the 5 mice tested. The glucan with $\overline{DP}_n$ 540 showed high activity by i.v. or i.p. administration (inhibition ratios, almost 100%), but it had low activity by s.c. administration (inhibition ratio, 63%). Administration p.o. of this glucan was almost ineffective, even at the high dose of 1500 mg/kg.

Direct Cytocidal Action of the Glucan on Tumor Cells. That we might examine whether the glucan with $\overline{DP}_n$ 540 had a direct cytocidal effect on tumor cells, ascites tumor cells of Sarcoma 180 were cultured in a medium containing concentrations of 0.5 to 2.5 mg glucan per ml. The viability of the tumor cells was examined by trypan blue staining and was found to be 98%, which did not differ from that of the cells cultured without addition of the glucan. It is clear that this glucan had no direct cytotoxicity against the tumor cells.

DISCUSSION

It was unclear whether or not linear (1 → 3)-β-D-glucans showed antitumor activity. For clarification we examined antitumor activity for a polysaccharide established to be a linear polymer of (1 → 3)-linked β-D-glucose residues, with no other linkages. The polysaccharide used in the present study is composed entirely of β-D-(1 → 3)-glucosidic linkage (5, 11, 14). The present results show that insoluble (1 → 3)-β-D-glucans with $\overline{DP}_n > 50$ had a strong antitumor activity and that the antitumor effect of the glucan with $\overline{DP}_n$ 50 in the pretreatment experiment was exquisitely schedule sensitive. Thus, the minimum molecular size of (1 → 3)-β-D-glucan required for the appearance of antitumor activity by i.p. administration appears to be about $\overline{DP}_n$ 50.

A possible reason for the apparent discrepancy of the efficacy of the glucan with $\overline{DP}_n$ 50 at specified time intervals prior to the transplantation of Sarcoma 180 cells is that glucan stays in the body of mice, in which it exerts its effect, according to its molecular weight. This is more important for the appearance of antitumor activity than it is for posttreatment.

In addition to molecular weight, which plays a dominant role in the antitumor activity of polysaccharide, the potential contribution of conformational structure of polysaccharide to antitumor activity had been suggested by our previous studies (16, 18), which dealt with the elucidation of conformation required for antitumor activity by use of absorption maximum shift with Congo red in the presence of lentinan, a branched (1 → 3)-β-D-glucan from *Lentinus edodes* (1, 2, 17), and also by use of ^{13}C nuclear magnetic resonance. Accordingly, identification of the ordered conformation as well as further elucidation of detailed structure seem to be very important in understanding the mode of action of antitumor polysaccharides. The present result indicates that well-defined glucans with $\overline{DP}_n > 50$, which are known to have an ordered conformation by ^{13}C nuclear magnetic resonance studies (15) and by absorption maximum shift studies with Congo red (12), had a strong antitumor activity. This further supports the foregoing conclusion.

Such a participation of supermolecular structure as well as the presence of a characteristic optimal dose for the antitumor activity of the glucan (Chart 1) and the absence of any direct cytocidal action against tumor cells suggest that there must be some mechanism in the biological system, which recognizes polysaccharide conformation, important for biological activity. The glucan with $\overline{DP}_n$ 16 (which may not be called polysaccharide) showed high antitumor activity by i.v. administration. This may be of special interest because antitumor polysaccharides can change the α helix of bovine serum albumin (4) or they can inactivate the third component of the complement *in vitro* (13), together with a transitory and marked increase in serum protein components (7). Despite the paucity of direct evidence, one interpretation for these findings may be that such substances, which would interact with polysaccharides, exist in mouse serum. It is therefore tempting to speculate that i.v. administration of such a glucan ($\overline{DP}_n$ 16) brings it to the target at which the substance(s) in the animal body interacts directly with the polysaccharide. This is in contrast with i.p. or s.c. administration in which such a low-molecular-weight glucan may be hydrolyzed into inactive smaller molecules before interacting with such substance(s).

The antitumor effect of this polysaccharide is considered to be host mediated by defense mechanisms of the host on the basis of absence of any direct cytocidal effect on tumor cells *in vitro* and on the effectiveness of pretreatment of animals by injection before transplantation of a tumor. The inhibition ratios observed with Ehrlich carcinoma, NTF reticulum cell sarcoma, and CCM adenocarcinoma were somewhat less than the high values achieved against Sar-

coma 180, but they were still significant. This implies that the immune capability against these transplantable tumors may be sufficiently strengthened by this glucan to bring about tumor rejection. On the other hand, Tokuzen and Nakahara (19) reported that antitumor polysaccharides gave the negative result on an autochthonous tumor, a spontaneous mammary adenocarcinoma of mouse. Consequently, one would not expect them to be effective in single use for human cancer. Interestingly, Kasamatsu *et al.* (6) reported that, in the radiation therapy of uterine cervical cancer, the combined use of PS-K (22), a glucan from the mycelium of *Coriolus versicolor*, showed a marked improvement. Polysaccharides might become effective against human cancer, used in conjunction with surgical operation, radiation therapy, suitable chemotherapy, and other immunopotentiators.

The active polysaccharide reported in this paper is the most reliable, in its purity and chemical structure, of the antitumor polysaccharides reported thus far. This makes it a potent tool for investigation of the mechanism of antitumor action and to provide ideas on a more potently active polysaccharide for future synthesis.

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