Conformation-Dependent Change in Antitumor Activity of Linear and Branched (1→3)-β-D-Glucans on the Basis of Conformational Elucidation by Carbon-13 Nuclear Magnetic Resonance Spectroscopy

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Received October 11, 1991

The antitumor activity of (1→3)-β-D-glucans was tested in order to clarify its conformation-dependent response together with conformational elucidation by carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy. It was shown that the following three conformations, single chain, single helix and triple helix, are readily distinguished by the high-resolution solid-state ^{13}C-NMR method. It turned out that preparations of linear (1→3)-β-D-glucans of a triple helical conformation were ineffective in the inhibition of tumor growth. These linear (1→3)-β-D-glucans were converted to an effective form in the inhibition of tumor growth when they were lyophilized from dimethyl sulfoxide (DMSO) solutions as a result of a conformational change from the triple helical to the single chain forms. They were not effective, however, when assayed in DMSO solution. In contrast, it was found that a branched (1→3)-β-D-glucan is effective not only in either saline solutions of the triple helical sample or the lyophilized sample from DMSO, but also in DMSO solution. The aforementioned drastic change in antitumor activity was interpreted in terms of resulting conformational changes as analyzed by the ^{13}C-NMR method.

Keywords antitumor activity; antitumor polysaccharide; confomation–activity relationship; conformational change; triple helix; single helix; random coil; linear (1→3)-β-D-glucan; branched (1→3)-β-D-glucan; ^{13}C-NMR

Introduction

It has been reported that (1→3)-β-D-glucans, which are widespread in nature, participate in various types of biological responses such as the host–defense mechanisms of plants and insects: promoting the production of phytoalexin,[2)] activation of prophenol oxidase,[3)] coagulation factor G of Limulus[4,5)] and a (1→3)-β-D-glucan binding protein of crayfish.[6)] In mammals, some (1→3)-β-D-glucans take the initiative in the development of a host–defense mechanism through the activation of an alternate pathway[7)] and recognition of monocyte β-glucan receptors.[8)] In addition, many studies have attempted to correlate the chemical structures of (1→3)-β-D-glucans with host-mediated antitumor activity.[9,10)]

X-Ray diffraction studies revealed the presence of a triple-helical conformation for linear (1→3)-β-D-glucans such as paramylon and the annealed fiber of curdlan.[11)] For a branched (1→3)-β-D-glucan, schizophyllan, it was also concluded that the glucan dissolved in water takes the triple helix conformation, as determined by viscosity measurements *etc.*[12)] Nevertheless, we showed that two additional forms, a single chain and single helix, can be distinguished by high-resolution carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy,[13,14)] although these two forms give rise to a halo diffraction pattern of X-ray diffraction. In particular, the single helical conformation is a dominant form in a resilient gel of curdlan, as viewed from conventional and high-resolution solid state ^{13}C-NMR.[13,14)] This means that the annealing of a hydrated sample at a temperature above 150 °C is essential to convert the linear glucans from the single helix to the triple helix, although such conversion is readily achieved by dissolution in an aqueous media in the case of branched glucans.[14a)]

Recently, we have pointed out that some of the above-mentioned biological responses, other than antitumor activity, are strongly related to their respective conformations.[5)] The triple helical (1→3)-β-D-glucans are unable to activate a Limulus coagulation factor G *in vitro*, whereas the single helical forms are strongly effective.[5)] In addition, we found that annealed curdlan with the triple helical conformation barely inhibited growth of solid tumor *in vivo*.[5)]

In this connection, it is worthwhile to examine the antitumor activity of various types of (1→3)-β-D-glucans, whose conformations are simultaneously characterized by high-resolution solid-state ^{13}C-NMR spectroscopy, to gain a uniform view of the conformation–activity relationship. This approach, using ^{13}C-NMR spectroscopy, is advantageous for this purpose, because we are readily able to determine a conformation of the starting materials prior to the bioassay experiments. Here, we aimed to analyze the antitumor effects of both linear and branched (1→3)-β-D-glucans and their conformation–activity relationships.

Results

Conformational Elucidation of (1→3)-β-D-Glucans by ^{13}C-NMR Spectroscopy Prior to an assay of antitumor activity, we examined the conformations of starting materials by high-resolution solid-state ^{13}C-NMR spectroscopy. It was straightforward to distinguish a single chain form from a triple helix by examination of the C-3 ^{13}C peak at 89 and 86 ppm, respectively.[13,14)] As demonstrated in Fig. 1 and 2, it is obvious that the triple helical forms (C-3b peak) of curdlan and HA β-glucan, which is isolated from the edible mushroom *pleurotus ostreatus*, are converted to the single chain forms (C-3a peak) as a result of lyophilization from a dimethyl sulfoxide (DMSO) solution, respectively. It is interesting that the single chain form of HA β-glucan is converted again to the triple helical form after dialysis in an aqueous solution for 24 h. We also found that the triple helical form of paramylon[14b)] is converted to the single chain form after lyophilization from a DMSO solution (spectra not shown).

Biological Activity Depending upon Conformations of (1→3)-β-D-Glucans In Table I, we summarized the data of antitumor activity together with the conformations of starting materials as revealed in this work and our previous

findings.[13,14] In the conventional assay of administration for 10 d, it was shown that paramylon lyophilized from DMSO solution significantly inhibited tumor growth, although the native paramylon granule lacked antitumor activity (Table I). In a similar manner, the lyophilized preparation of annealed curdlan from DMSO solution (degree of polymerization (d.p.) 114) was able to strongly inhibit tumor growth *in vivo*, although the potency of the annealed curdlan at 160 °C was greatly suppressed (Table I).

Antitumor Test by Single Administration of a Sample We tried other assays by single injection of a sample, followed by weighing the tumors, on a variety of days, as shown in the columns of administration and assessment in Table II. It was found that HA β-glucan administered on day 7 suppressed tumor growth by an inhibition ratio (I.R.) of 94% as compared with the control group on day 18.

Hereafter, we used the following assay schedule: solutions of samples were injected only on day 7 in tumor bearing mice and the tumors were weighed on day 21.

Potency of (1→3)-β-D-Glucans Depending on the Dose in Saline It was found that HA β-glucan and HA β-glucan lyophilized from DMSO solution were equally effective with a dose of 1 mg/kg in inhibiting of tumor growth, according to the assay method of single administration. However, Table III shows that both paramylon and curdlan lyophilized from DMSO solutions required doses of 10 mg/kg.

Potency of (1→3)-β-D-Glucans in DMSO Solution We tried an assay of dissolved (1→3)-β-D-glucans in DMSO by the use of single administration. In a single injection a DMSO solution of 0.05 ml/mouse was nontoxic to mice.

It was found that linear (1→3)-β-D-glucans in DMSO

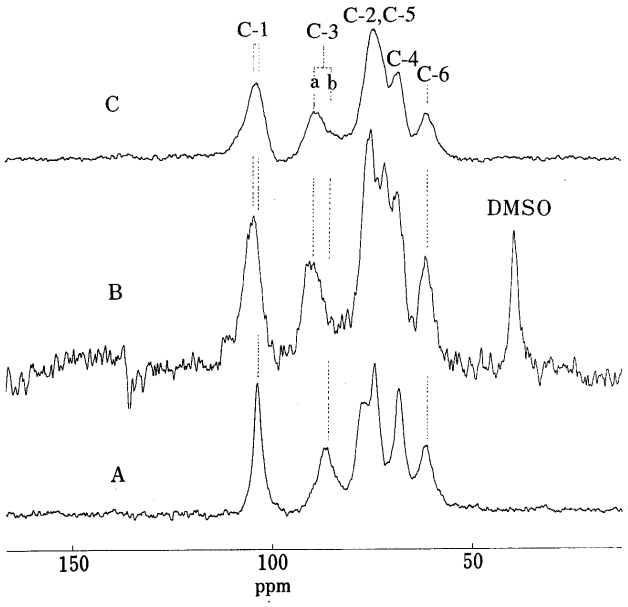

Fig. 1. High-Resolution Solid-State ^{13}C-NMR Spectra of Curdlan from Various Preparations

A. Annealed curdlan at 180 °C for 10 min. B. Annealed curdlan at 160 °C for 1 h followed by lyophilization from DMSO solution. C. Curdlan powder as received.

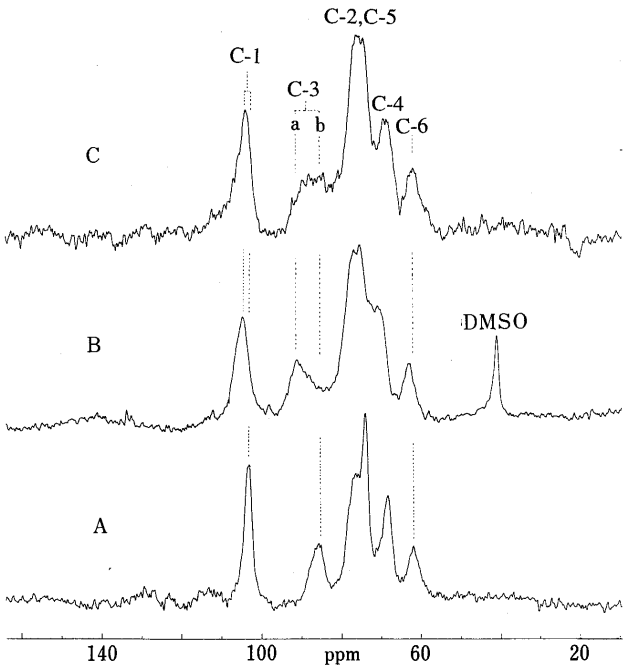

Fig. 2. High-Resolution Solid-State ^{13}C-NMR Spectra of HA β-Glucan from Various Preparations

A. Lyophilized from aqueous solution. B. Lyophilized from DMSO solution. C. Sample B was dialyzed against water for 24 h, followed by lyophilization.

TABLE I. Change of Antitumor Activities of Linear (1→3)-β-D-Glucans Depending upon Their Conformations

Sample	Conformation of starting material	Total dose[a] (mg/kg/10 d)	Tumor weight[b] Mean±S.E. (g)	p[c]	Inhibition ratio (%)	Complete regression[d]
Control			6.82±1.97			0/6
Paramylon (d.p. 700)	Triple helix	10	4.19±0.96		39	0/6
		50	12.90±2.33	<0.1	−89	0/6
Control			8.58±1.78			
Paramylon lyophilized from DMSO	Single chain	10	0.90±0.87	<0.005	90	4/6[d]
		100	0.04±0.04	<0.001	100	5/6[d]
Control			9.31±2.29			0/6
Curdlan annealed at 160° for 1 h (d.p. 119)	Triple helix	10	3.38±1.40	<0.1	64	0/6[e]
		100	6.36±2.71		32	0/6
Annealed curdlan lyophilized from DMSO (d.p. 114)	Single chain	10	0.83±0.79	<0.01	91	4/6[d]
		100	0.04±0.04	<0.01	100	5/6[d]
Curdlan lyophilized from DMSO (d.p. 3980)	Single chain	10	1.02±1.02	<0.01	89	5/6[d]

a) Mice were injected i.p. with sample in saline (0.25 ml/mouse) daily for 10 d. *b*) Tumors were weighed after 5 weeks. *c*) Student's *t*-tests of significance for differences between tumor weights in treated group and those in control group by using two sides. *d*) The difference in the data and control was regarded as significant ($p<0.05$) by using the one tail test of Fisher's exact test. *e*) One of 6 mice died on day 33.

TABLE II. Therapeutical Process According to Single Administration Schedule of HA β-Glucan, a Branched (1→3)-β-D-Glucan

	Administration[a]	Assessment	Tumor weight Mean±S.E. (g)	p^{b}	Inhibition ratio (%)	Complete regression
Control group		On day 7	0.13±0.02			0/5
		8	0.10±0.01			0/5
		12	0.30±0.06			0/5
		15	0.44±0.10			0/5
		18	0.33±0.12			0/5
		19	0.76±0.12			0/5
		28	1.54±0.36			0/5
Treated group	On day 1	7	0.06±0.01	<0.05	54	0/5
	1	8	0.06±0.01	<0.05	40	0/5
	1	12	0.06±0.01	<0.001	80	0/5[c]
	7	8	0.13±0.02		−30	0/5
	7	12	0.08±0.02	<0.01	73	0/5
	7	18	0.02±0.01	<0.05	94	2/5
	14	15	0.15±0.02	<0.05	66	0/5
	14	19	0.10±0.02	<0.001	87	0/5
	14	28	0.20±0.11	<0.01	87	0/5

a) Mice were injected i.p. only once with sample in saline by one dose of 10 mg/kg. *b*) Student's *t*-tests of significance for differences between tumor weights in treated group and those in control group by using two sides. *c*) One of 5 mice died on day 9.

TABLE III. Antitumor Activity of (1→3)-β-D-Glucans in Saline by Administration Only Once on Day 7 then Assessment on Day 21

Sample	Conformation of starting material	Dose (mg/kg)	Tumor weight Mean±S.E. (g)	p^{a}	Inhibition ratio (%)	Complete regression[b]
Control			1.82±0.24			0/6
Paramylon lyophilized from DMSO	Single chain	0.5	1.13±0.41		38	0/6
		1.0	1.51±0.37		17	0/6
		10.0	0.47±0.23	<0.005	74	2/6
Control			1.89±0.58			0/6
Curdlan lyophilized from DMSO	Single chain	1.0	1.00±0.30		47	0/6
(d.p. 3980)		10.0	0.19±0.08	<0.05	90	1/6
Curdlan	Single chain/triple helix	1.0	1.99±0.44		−6	0/6
(d.p. >4000)	(*ca.* 90%) (*ca.* 10%)	10.0	1.40±0.50		26	0/6
Curdlan annealed at 180° for 10 min	Triple helix	1.0	0.69±0.19	<0.1	63	0/6
(d.p. 106)		10.0	2.48±0.92		−31	0/6
Control			2.49±0.99			0/6
HA β-glucan	Triple helix	0.5	1.58±0.77		37	0/6
		1.0	0.54±0.24	<0.1	78	2/6
		10.0	0.04±0.03	<0.05	98	4/6[b]
HA β-glucan lyophilized from DMSO	Single chain	0.5	1.70±0.97		32	3/6
		1.0	0.47±0.16	<0.1	81	1/6
		10.0	0.19±0.04	<0.05	92	2/6

a) Student's *t*-tests of significance for differences between tumor weights in treated group and those in control group by using two sides. *b*) The difference in the data and control was regarded as significant ($p<0.05$) by using the one tail test of Fisher's exact test.

TABLE IV. Antitumor Activity of (1→3)-β-D-Glucans in DMSO by Single Injection (0.05 ml/Mouse)[a]

Sample	Conformation of starting material	Dose (mg/kg)	Tumor weight Mean±S.E. (g)	p^{b}	Inhibition ratio (%)	Complete regression[c]
Control in DMSO			2.21±0.67			0/6
Paramylon in DMSO	Random coil	1.0	1.81±0.33		18	0/6
		10.0	2.05±0.56		7	0/6
Control in DMSO			1.42±0.45			0/6
Curdlan in DMSO (d.p. 3980)	Random coil	1.0	1.35±0.54		5	1/6
		10.0	1.30±0.31		9	0/6
Control in DMSO			2.24±0.79			0/6
HA β-glucan in DMSO	Random coil	0.5	3.28±0.91		−46	0/6
		1.0	0.64±0.29	<0.1	71	1/6
		10.0	0.30±0.23	<0.05	87	4/6[c]

a) Mice were injected i.p. with a sample in DMSO on day 7, then tumors were weighed after 3 weeks. *b*) Student's *t*-tests of significance for differences between tumor weights in treated groups and those in control group by using two sides. *c*) The difference in the data and control was regarded as significant ($p<0.05$) by using the one tail test of Fisher's exact test.

turned out to be ineffective. By contrast, HA β-glucan in DMSO was effective even with a dose of 1 mg/kg in the inhibition of tumor growth, as shown in Table IV. As described above, the conformation of HA β-glucan was again converted to the triple helix after dialysis of this DMSO solution against water for 24 h (Fig. 2C). It appears that HA β-glucan, initially taking a random coil form in DMSO, is first converted to the single helical conformation within 24 h *in vivo*, followed by the formation of the final triple helical form.

Discussion

Biologically Active Conformations of (1→3)-β-D-Glucans We found that paramylon preparation lyophilized from a DMSO solution exhibits antitumor activity, although the native paramylon granule is inactive (Table I). This finding is consistent with our previous observation: curdlan lyophilized from DMSO solution exhibits an antitumor activity, while the annealed curdlan sample resulted in inactivity.[5] These findings were well explained in that the single chain preparations, such as the lyophilized product from a DMSO solution, are more effective in the inhibition of tumor growth than the triple helical samples such as paramylon granule or annealed curdlan.[13] We emphasize that the samples were assayed without sterilization in an autoclave, to prevent an avoidable conformational change due to heating. This is the reason the single chain-curdlan is effective in the present assay. It is worthwhile to point out here that the anhydrous sample of the single chain form is readily converted to the single helical conformation by either exposure to high humidity or suspension in aqueous solution.[14] Thus, it is conceivable that the single chain form of the starting materials are converted to the single helical form in saline. In other words, an active conformation of linear (1→3)-β-D-glucans *in vivo* is the single helix. Thus, any confomational change to the triple helix from other forms would result in a loss of the antitumor activity, as observed for the annealed curdlan.

It was previously shown that the above-mentioned helical conformation is depending on molecular weight.[13b] Therefore, it is also important to take into account of the effect of a plausible change in molecular weight during the annealing of a polysaccharide, since a severe thermal degradation of curdlan was accompanied by the d.p. 3980 to 106 (or 119) as a result of heating at 180 °C for 10 min (or at 160 °C for 1 h). It is mentioned that antitumor activity could be completely lost when d.p. of a linear (1→3)-β-D-glucan is less than 38, as in the case of laminaran.[5] To evaluate the effect of depolymerization by annealing, we examined the antitumor activity of this depolymerized curdlan dissolved in DMSO, followed by lyophilization (Table I). It is clear that the antitumor activity is restored by this procedure, in spite of the depolymerization up to d.p. 114. The present NMR study shows that this depolymerized sample takes the single chain form after lyophilization from DMSO solution (Fig. 1). Thus, it seems that the single chain form of the linear (1→3)-β-D-glucan, whose d.p. is higher than 38, is essential to be able to inhibit tumor growth *in vivo*, while the triple helix is ineffective for the antitumor activity. We observed also *in vitro* that the annealed curdlan lyophilized from DMSO solution responded sensitively to factor G of Limulus, at which a half-maximal response was obtained in the region of 1—10 ng/ml, but the annealed curdlan did not, by the method already described.[5] It was further demonstrated that the triple helix is ineffective for the activation of factor G. These facts *in vivo* and *in vitro* are analogous to previous findings: an attack of acid or enzyme to curdlan gel is significantly decreased with heating at 120 °C.[15] Therefore, it is concluded that the single chain form as a starting form is more effective than the triple helical form for both antitumor activity and activation of factor G in the Limulus test.

Assay by Single Injection The above-mentioned antitumor test is excellent in the reproducibility of data as compared with data of single injection. The assay by single injection has two merits. The first is advantageous for an assay in solvents other than saline, since the single injection permits one to perform the level of administration in a minimum volume to avoid harmful effects of the solvent. The second is that any conformational change in the sample during the course of study, and errors in weighing of a minute dose of samples, are kept minimal.

Initial trials of single injection in a variety of schedules were summarized in Table II. We tried the assay by administration of HA β-glucan, since the branched (1→3)-β-D-glucan is highly inhibitory to tumor-growth and advantageous for higher solubility than other (1→3)-β-D-glucans in water.[10] The data showed that the inhibition ratio (94%) on day 18 after administration on day 7 was close to the potency (I.R. of 99.9%) achieved by conventional assay in the previous paper.[5] Thus, the assay method of single injection was fixed on the schedule of administration at day 7 and of assessment at 3 weeks.

Two saline suspensions of paramylon and curdlan lyophilized from DMSO solutions exhibited growth inhibition of tumors with a dose of 10 mg/kg by single injection (Table III). These potencies were consistent with the activities of the total dose of 10 mg/kg by the conventional assay in Table I. In the latter assay, the stocked suspensions of samples were administered daily in the dose of 1 mg/kg/d for 10 d. This means that an active form in saline is maintained throughout 10 d. In fact, the single helix arising from a single chain sample is very stable in aqueous media for a linear (1→3)-β-D-glucan with a molecular weight higher than d.p. 40, as examined in our ^{13}C-NMR studies.[14b] In addition, Okuyama *et al.* showed that the single helical conformation is retained in curdlan as studied by X-ray diffraction.[16] That is, the antitumor activity of linear (1→3)-β-D-glucans depends on whether the conformation of samples is the single chain/single helix or triple helix.

On the other hand, it is interesting to note that HA β-glucan was effective with either sample, the triple helix or single chain, with a dose of 1 mg/kg. The triple helical form of branched (1→3)-β-D-glucans was readily converted to the single helix under slightly alkaline conditions (<0.1 M),[17] due to these loosely-formed triple stranded helices[12] and fixation of the side chains in association with water molecules.[18] Thus, it is likely that the triple helical conformations of branched glucans might be partially dissociated to the single helix to some extent *in vivo*, in contrast to the case of the rigid triple helical forms of

curdlan.[11b,19] In fact, a considerable quantity of the single helical forms was observed in the brittle gel of schizophyllan prepared by heating,[5] and in the soft gel of HA β-glucan prepared by 0.05 M NaOH,[13c] as viewed from the high-resolution solid state and the conventional ^{13}C-NMR, respectively. In addition, lentinan lyophilized from aqueous solution gave rise to the single chain conformation.[14a,20] These observations indicated that the single helical conformation is also stable in branched (1→3)-β-D-glucans.

DMSO Solution as a Single Injection When (1→3)-β-D-glucans dissolved in DMSO were administered with a single injection of 0.05 ml/mouse on day 7, they exerted no toxic effect of DMSO in treated mice. It is surprising to note that the antitumor activity of linear (1→3)-β-D-glucans is substantially lost when dissolved in DMSO: from I.R. 90% to 7% of paramylon, and from I.R. 89% to 9% of curdlan with the dose of 10 mg/kg (Tables I and IV). This observation is consistent with previous findings that the antitumor activity of lentinan from *Leninus edodes* was lost in the presence of urea[21a] or in DMSO.[21b]

In contrast, we found that HA β-glucan in DMSO inhibited tumor growth in the dose of 1 mg/kg, as well as in saline (Table IV). It was shown that DMSO as a solvent was excreted into mouse urine before 24 h. In order to mimic this conformational change of HA β-glucan *in vivo*, the DMSO solution was dialyzed against deionized water for 24 h. The resulting conformation turned out to be the triple helix, as judged from the characteristic C-3 peak-positions of the solid-state ^{13}C-NMR spectra (Fig. 2C). This finding suggests that an active form of HA β-glucan was derived from the randomly coiled form in a mouse abdominal cavity. This finding also parallels the fact that schizophyllan, injected with urea, inhibited tumor growth.[21a]

This data, however, is in conflict with the data of lentinan or curdlan with urea or in DMSO. It is true that lentinan is only slightly soluble and curdlan is barely soluble in an aqueous solution. Subsequently, it appears that these molecules from a randomly coiled form in a DMSO solution take an aggregated disordered form which is resistant to conversion into the single helix.

In conclusion, we found that an active conformation for the antitumor activity of (1→3)-β-D-glucans is the single chain/single helix, in spite of the previous belief that the triple helix conformation was best.

Experimental

Materials Paramylon, a linear (1→3)-β-D-glucan of d.p. 700, was provided by Professor B. A. Stone of Trobe University, Australia. A part of paramylon was dissolved in DMSO, followed by lyophilization. Curdlan, a linear (1→3)-β-D-glucan of d.p. > 4000, was purchased from Wako Pure Chemical Co., Osaka, Japan. Two samples of annealed curdlan were prepared by annealing a gel in a sealed glass tube in the presence of water at 180 °C for 10 min and 160 °C for 1 h, followed by slow cooling, respectively. A part of the latter annealed curdlan was dissolved in DMSO and lyophilized. HA β-glucan of d.p. 270, a branched (1→3)-β-D-glucan isolated from edible mushroom *Pleurotus ostreatus* (Fr.) Quél., was lyophilized from an aqueous solution as previously published.[10] HA β-glucan lyophilized from DMSO was also prepared from this DMSO solution.

General Procedure The average degree of polymerization of the samples was estimated from the peak position on size exclusion chromatography using TSK gel (Tosoh Co., Tokyo) with 0.3 M NaOH as a mobile phase and pullulans as a molecular weight standard. 75.46 MHz ^{13}C-NMR spectra were recorded on a Bruker CXP-300 spectrometer under a condition of cross-polarization-magic angle spinning. Samples were contained in a rotor of zirconia and spun as fast as 3 kHz. ^{13}C chemical shifts were referred to tetramethylsilane through the carboxyl peak of glycine (176.03 ppm).

HA β-glucan dissolved in DMSO (200 mg/3 ml) was enclosed in Visking tube (0.6 × 20 cm), dialyzed against deionized water (5 l × 2) for 24 h, and the resulting soft gel was lyophilized. Conformational change was judged by high-resolution solid-state ^{13}C-NMR, as described above.

Bioassay of the Antitumor Activity Ascites of sarcoma 180 (5×10^6 cells) were transplanted subcutaneously into female mice of the CLJ-ICR strain. Samples were administered intraperitoneally to tumor-bearing mice. Two schedules of assay were practiced. One assay was performed conventionally by the schedule described previously.[10] Homogeneous suspensions/solutions were prepared by grinding (1→3)-β-D-glucan samples in saline, followed by refrigeration without sterilization in an autoclave. The stocked solution/suspension (0.25 ml/d/mouse) was injected for 10 d, beginning the day after implantation of the tumor, and the tumors were weighed at the end of 5 weeks. Complete regression was defined as when a mouse was tumor-free or had a scar weight less than 0.1 g.

An alternative schedule made use of single administration on day 7 at 6-d intervals after tumor-inoculation. Tumors were then weighed at the end of 3 weeks. Both saline and DMSO samples were prepared following lyophilization from DMSO just before their use, then mice were injected with a saline solution (0.25 ml/mouse) and a DMSO solution (0.05 ml/mouse). Prior to setting the schedule, several therapeutic processes were tested in a variety of schedules, as summarized in Table II.

Acknowledgment The authors are grateful to Dr. J. Aketagawa and Dr. Y. Shibata of Seikagaku Corporation for performing assays of the activation of factor G of Limulus and for measurement of molecular weight. We also thank Dr. A. Hoshi of our Institute, for helpful suggestions.

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