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Relationship between conformation and biological response for (1→3)-β-D-glucans in the activation of coagulation Factor G from limulus amebocyte lysate and host-mediated antitumor activity. Demonstration of single-helix conformation as a stimulant.

Hazime Saitô*,‡ Yûko Yoshioka†, Nobuaki Uehara†,
**Biophysics and †Chemotherapy Divisions, National Cancer Center Research Institute, Tsukiji 5-chome, Chuo-ku, Tokyo 104 (Japan)*

Jun Aketagawa, Shigenori Tanaka, and Yûkô Shibata
Tokyo Research Institute, Seikagaku Corporation Ltd., Tateno 3-chome, Higashiyamato, Tokyo 189 (Japan)

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ABSTRACT

The relationship between the conformation of (1→3)-β-D-glucans in gel or hydrated form and the stimulation of two types of biological responses, namely, activation of coagulation Factor G from limulus amebocyte lysate (LAL) and host-mediated antitumor activity was examined. Both types were activated by the single-helical conformation, as revealed by high-resolution, solid-state ^{13}C-n.m.r. spectroscopy. The potency of activation of Factor G was increased over 100-fold by treatment with a NaOH solution which leads to a complete or partial conversion from the triple to the single helix. Such a single-helix specific response was also demonstrated for the antitumor activity of curdlan, although the distinction was less pronounced for branched (1→3)-β-D-glucans. The presence of the single-helix conformation was observed in schizophyllan gel, even though the triple helix is the most stable form of branched glucans in aqueous media.

INTRODUCTION

In plants and animals, including humans, (1→3)-β-D-glucans are involved in a variety of host-defense biological responses, such as host-mediated antitumor activity[1–5], activation of complement C3 by way of the alternative pathway[6,7], activation of the coagulation system of horseshoe crab amebocyte lysate (LAL)[8], activation of polyphenol oxidase system of insect[9,10], activation of defense gene to pathogen attacks in plant[11], and recognition of human monocyte β-D-glucan receptor[7,12]. Some of these biological responses appear to be activated by a recognition of a certain region of ordered conformation in the D-glucans, because low-molecular-weight D-glucans were ineffective both in antitumor activity[13,14] and in activation of Factor G in LAL[15]. Furthermore, the antitumor activity of lentinan, a branched (1→3)-β-D-glucan, was lost when the ordered conformation was disrupted by urea or dimethyl sulfoxide[16,17].

‡ Present address: Department of Life Science, Himeji Institute of Technology, Harima Science Garden City, Kamigori-cho, Hyogo, 678-12, Japan.

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Three conformations, single chain, single helix, and triple helix[21–28], are observed for (1→3)-β-D-glucans of higher molecular-weight in the solid state, depending on the sources, methods of isolation, and physical treatments, as evidenced from the conformation-dependent displacement of ^{13}C-n.m.r. peaks (up to 8 p.p.m.)[18–20] in high-resolution, solid-state ^{13}C-n.m.r. spectroscopy[21–28]. Interconversion between these conformations takes place under controlled treatment[23,25–27]. In particular, the single-chain form is reversibly converted to and from the single-helix form by hydration and dehydration. The triple-helix form is obtained[23] either by annealing curdlan at a temperature above 150°, followed by slow cooling, or by dissolving a branched D-glucan in aqueous media[24,27]. The triple-helix form is readily converted to the single-chain form by dissolution in dimethyl sulfoxide[25] or alkaline solution, followed by neutralization or lyophilization, (or both). Thus, it was possible to study which type of conformation stimulates more strongly the aforementioned biological responses, and we chose the activation of Factor G in LAL and the antitumor activity as typical *in vitro* and *in vivo* systems.

EXPERIMENTAL

Materials. — Curdlan[29] was purchased from Wako Pure Chemical Industries, Inc. (Osaka, Japan). Annealed curdlan was prepared by annealing a curdlan gel in a sealed glass tube in the presence of water at 180°, followed by either rapid cooling in ice–water or slow cooling by keeping the sample at ambient temperature[29]. Lyophilized curdlan was obtained by lyophilization of a dimethyl sulfoxide solution. Laminaran from *Laminaria* species (Nutritional Biochemistry Corporation, Ohio, U.S.A.) was purified by the procedure described previously[26]. HA-β-glucan[4] and schizophyllan[2,30] were used as typical branched glucans; schizophyllan was obtained from commercial "Sonifilan®" (Kaken Pharmaceutical Co., Ltd., Tokyo, Japan) and has a weight-average mol. wt. of 450 000. HA-β-glucan and schizophyllan were obtained by lyophilization of a dimethyl sulfoxide solution as described earlier for the preparation of lyophilized curdlan.

Treatment of curdlan, schizophyllan[2], and HA-β-glucan. — The annealed curdlan described above was dissolved in 0.3M NaOH solution for 10 min, followed by neutralization with a 0.3M HCl solution. The resulting gel samples were used for the assay of activation of Factor G and they were lyophilized for n.m.r. spectroscopy.

To a solution of Sonifilan® (100 mg) in saline solution (10 mL; 5 vials) was added 2M NaOH (2.5 mL; final concentration, 0.4M). The mixture was kept on ice for 90 sec, the base neutralized by addition of 2M HCl, and the solution dialyzed against distilled water. NaCl-treated schizophyllan was prepared as a control by use of a similar procedure replacing NaOH and HCl with M NaCl. For n.m.r. spectroscopy, hydrated schizophyllan was prepared by placing a sample lyophilized from the dimethyl sulfoxide solution in a desiccator at a relative humidity of 96% for over 12 h. The schizophyllan gel was prepared by dissolving the sample lyophilized from the dimethyl sulfoxide solution in an aqueous medium at an elevated temperature (>80°), followed by cooling.

An aqueous solution of HA-β-glucan was treated with NaOH solution, followed by neutralization.

Characterization of the conformation by high-resolution, solid-state ^{13}C-n.m.r. spectroscopy. — High-resolution, solid-state ^{13}C-n.m.r. spectra were recorded with a Bruker CXP-300 spectrometer, equipped with an accessory for cross-polarization–magic-angle spinning (CP–MAS). The samples were contained in a ceramic rotor and spun as fast as 3 kHz. Duration of 90° pulse, contact time, and repetition times were 3.5 μs, 1 ms, and 4 s, respectively. The spectra were accumulated more than 500 times and the ^{13}C-chemical shifts are referenced relative to the signal of external tetramethylsilane (through the peak of the carboxyl group of glycine, δ 176.03).

Activation of Factor G from limulus amebocyte lysate. — The amebocyte lysate from *Tachypleus tridentatus* (Japanese horseshoe crab belonging to *Limulidae* and very close to *Limulus polyphemus*) was used for this study[8]. The activation was assayed at 37° by measurement of amidase activity of the proclotting enzyme, induced by activated Factor G, acting on Boc-Leu-Gly-Arg-4-nitroanilide (*p*NA) as a substrate[8]. The reaction mixture contained the (1→3)-β-D-glucan sample; Factor G fraction (20 μL; A_{280} 0.9) and proclotting enzyme fraction (30 μL; A_{280} 2.5), both of which prepared as described previously[8]; Tris·HCl (20 μmol) buffer (pH 8.0); $MgCl_2$ (20 μmol); and Boc-Leu-Gly-Arg-*p*NA (0.13 μmol), in a total volume of 200 μL. It was incubated at 37° for 30 min. 4-Nitroaniline liberated was converted into an azo-dye by addition of 0.04% $NaNO_2$ in 0.48M HCl, 0.3% ammonium sulfamate, and 0.07% *N*-(1-naphtyl)ethylenediamine dihydrochloride (0.5 mL each), and the absorbance at 545 nm was determined. The samples tested were dissolved or suspended in water, and then diluted serially with distilled water unless otherwise noted. The glucan concentration of the sample was expressed as D-glucose determined by the phenol–H_2SO_4 method[31].

Bioassay of the antitumor activity. — Ascites of sarcoma 180 (5×10^6 cells) were transplanted subcutaneously into female mice of the CLJ-ICR strain. Administration of samples and assessment of results were performed by the method described previously[4] as follows. A sample of 10 or 1 mg/kg was suspended homogeneously in saline solution (2.5 mL per mouse), 1/10 of the solution (or suspension) was injected intraperitoneally daily for 10 days, starting the day after transplantation of the tumor, and the results were assessed at the end of five weeks.

RESULTS AND DISCUSSION

Conformation of (1→3)-β-D-glucans. — It was essential to ascertain the conformation of (1→3)-β-D-glucans in the hydrated state in order to establish a relationship between conformation and biological response,as will be shown below. The sodium hydroxide-induced conformational change of (1→3)-β-D-glucans was also studied in order to explain the data for activation of Factor G from LAL. For this purpose, the ^{13}C-n.m.r. spectrum of lyophilized gel (xerogel) was also analyzed because the C-3 ^{13}C-n.m.r. peak from the single chain (converted from the single helix by dehydration) is well separated from that of the triple helix[26].

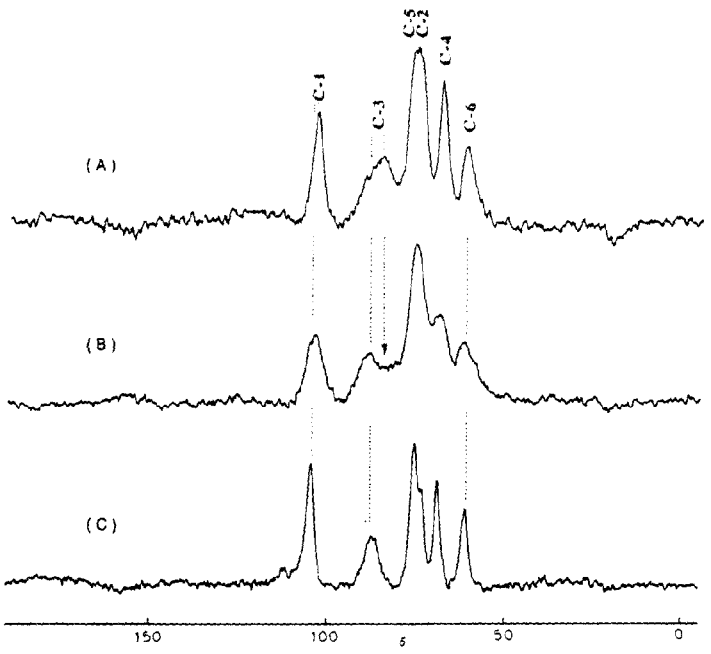

Fig. 1. ^{13}C-N.m.r. spectra of curdlan annealed at 180° and then slowly cooled (A); of its sodium hydroxide-treated sample, neutralized, and lyophilized (B); and of sodium hydroxide-treated curdlan powder, neutralized, and lyophilized, as a control (C). The arrow indicates the position of the peak corresponding to the triple helix.

The high-resolution, solid-state ^{13}C-n.m.r. spectra of curdlan, annealed at 180° and then slowly cooled (Fig. 1, A); of its sodium hydroxide-treated sample, neutralized and lyophilized (Fig. 1, B); and of sodium hydroxide-treated curdlan powder, neutralized and lyophilized (Fig. 1, C) showed a major conformational change from the triple helix (Fig. 1, A) to the single helix (Fig. 1, B) as evidenced by the presence of the single chain signal at δ 89[25,26]. A smaller peak corresponding to the C-3 peak of the triple helix (δ 85, indicated by an arrow) remained in this case, as compared with the ^{13}C-n.m.r. spectrum of curdlan powder treated with a sodium hydroxide solution (Fig. 1, C), was due to the incomplete disruption of the tightly-held triple helix, even after treatment with 0.3M sodium hydroxide.

The ^{13}C-n.m.r. spectra of schizophyllan exhibiting different types of conformation are illustrated in Fig. 2. In particular, the presence of an unresolved C-3 shoulder at δ 89 (Fig. 2, A) is characteristic of the partial change in conformation of schizophyllan by sodium hydroxide treatment, followed by neutralization and dialysis, from the triple helix (sample lyophilized from an aqueous solution; Fig. 2, B) to the single-chain form (sample lyophilized from a dimethyl sulfoxide solution, Fig. 2, E); the proportion of triple helix as 60% and that of single chain as 40% was estimated from the relative peak area. As mentioned above, the single-helix conformation is formed by neutralization after sodium hydroxide treatment. The reverse change from the single helix to the triple

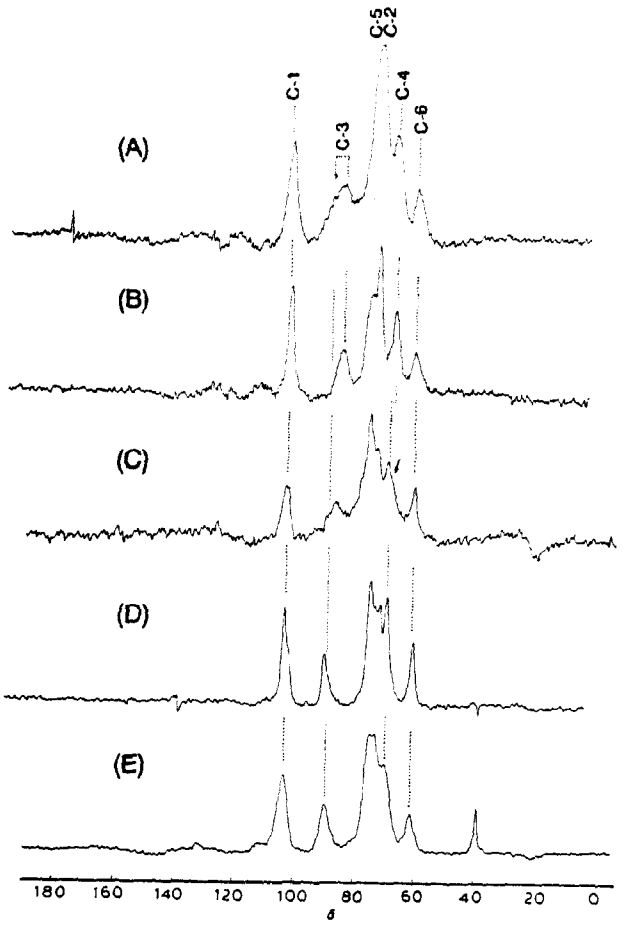

Fig. 2. ^{13}C-N.m.r. spectra of schizophyllan in the solid and gel states: (A) Sodium hydroxide-treated schizophyllan, neutralized, dialyzed, and lyophilized; (B) lyophilized sample from aqueous solution; (C) elastic gel; (D) lyophilized sample from a dimethyl sulfoxide solution, and then hydrated in a desiccator at 96% relative humidity; and (E) anhydrous sample lyophilized from a dimethyl sulfoxide solution. The arrow in (A) indicates the peak corresponding to the single-chain conformation. The arrow in (C) indicates the shoulder corresponding to the single helix conformation. A sharp peak at δ 39.5 in (E) was assigned to the methyl carbon atoms of dimethyl sulfoxide.

helix could occur for branched (1→3)-β-D-glucans during prolonged dialysis (> overnight)[27]. It is interesting to note that the single-chain conformation of schizophyllan was substantially stabilized by hydration (96% relative humidity), as shown by the significant peak-narrowing (Fig. 2, D). This stabilization did not occur in curdlan, because hydration caused a conformational change to the single helix form[26]. In addition, it is interesting that fully hydrated schizophyllan gel gave a composite spectrum of the single and triple-helix form in a 3:2 ratio, as estimated from the peak intensities of the C-4 signal and its high-field shoulder (indicated by an arrow, Fig. 2, C), rather than the triple-helix form which is known to be the most stable form for branched D-glu-

TABLE I

Conformations of (1→3)-β-D-glucans used in this study

Sample	*Gel*	*Hydrated by humidity, after lyophilization from a Me_2SO solution*	*Anhydrous*		
			Lyophilized from a Me_2SO solution	*Lyophilized from a NaOH solution*	*Lyophilized from an aqueous solution or a gel*
Curdlan	Single helix[a]	Single helix[b]	Single chain[c]	Single chain[d]	Single chain[e]
Schizophyllan	Single helix–triple helix[d,f]	Single chain[d]	Single chain[e]	Single chain–triple helix[d,g]	Triple helix[h]
HA-β-glucan	Triple helix[d,i]	Single chain[d]	Single chain[e]		Triple helix[h]
Laminaran	[i]	Single chain[e]	Single chain[e]		Triple helix[h]

[a] From ref. 27. [b] From ref. 26. [c] From ref. 25. [d] This work. [e] From ref. 22. [f] Relative proportion of single to triple, ~3:2. [g] Relative proportion of single chain to triple helix ~2:3. [h] From ref. 24. [i] Inferred from the data of aldehyde derivative. [j] Not gelable.

cans[24,27,28]. This observation is in contrast to that for lentinan gel, the conformation of which is composed mainly of the triple-helix form[27].

The conformational features of (1→3)-β-D-glucans thus obtained are summarized in Table I, together with the data previously published[21–28]. As mentioned in our previous paper[27], we were unable to record the ^{13}C-n.m.r. spectra of HA-β-glucan in the gel state because of instability of the magic-angle spinning. However, it is reasonable to infer its conformation from the data of the aldehyde derivative of HA-β-glucan, as shown in Table I. It is noteworthy that the gel network of schizophyllan consists in majority of the single helix (60%), the remaining being triple helix (40%), although the triple-helix form is preponderant in the anhydrous state obtained by lyophilization of an aqueous solution (Table I). This proportion of the triple-helix form as cross-links was sufficient to suppress completely the high-resolution ^{13}C-n.m.r. signals, as recorded by a conventional spectrometer, when the data of chemically cross-linked, synthetic polymers are taken into account[32,33]. This result also implies that the single-helix conformation of schizophyllan could be stable, depending on the mode of sample preparation, although similar branched D-glucans, such as lentinan and HA-β-glucan, are mainly in the triple-helix conformation in the gel state[27,28]. Actually, we found that schizophyllan gel exhibited an elastic property similar to that of curdlan (arising from the single-helix conformation), although gels of another branched (1→3)-β-D-glucans are either brittle or paste-like. Furthermore, it was recognized that hydration by humidity did not readily induce conformational change of branched (1→3)-β-D-glucans from the single chain to the single helix (Table I).

Activation of Factor G from LAL. — The plots of the potency (as expressed by ΔA_{545} after 30 min) of linear (Fig. 3) and branched (1→3)-β-D-glucans (Fig. 4), for activation of Factor G from LAL against concentration of the D-glucans showed clearly that only high-molecular-weight glucans were effective. Laminaran from *Laminaria*

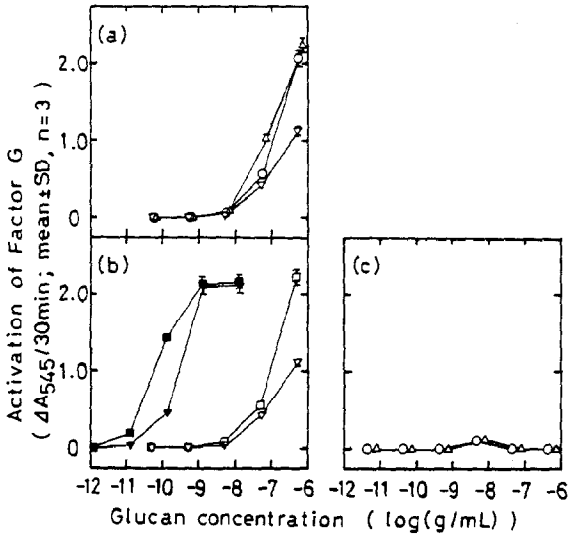

Fig. 3. Activation of coagulation Factor G from LAL by linear (1→3)-β-D-glucans. Except for the sodium hydroxide-treated samples, all samples were suspended in distilled water. Panel a: (○) Curdlan powder; (△) lyophilized curdlan from a dimethyl sulfoxide solution; and (▽) annealed curdlan at 180°, and then slowly cooled. Panel b: (▽) Curdlan annealed at 180° and then slowly cooled (sample 1); (□) curdlan annealed at 180°, and then rapidly cooled (sample 2); (▼) sample 1 dissolved in a 0.3M NaOH solution and diluted serially with the same solution, followed by neutralization; and (■) sample 2 dissolved in a 0.3M NaOH solution and diluted serially with the same solution, followed by neutralization. Panel c: Laminaran from *Laminaria* species, lyophilized from aqueous (○) and from dimethyl sulfoxide (△) solution. The results are means ± SD (standard deviation) of triplicate measurements. When not shown, the SD bars fall within the size of symbols.

species (d.p. ~38), obtained by lyophilization of either an aqueous or dimethyl sulfoxide solution, was ineffective (Fig. 3, c). Four curdlan preparations, *i.e.*, curdlan powder, a sample obtained by lyophilization of a dimethyl sulfoxide solution, and two annealed samples were effective, although the potency of the preparations varied substantially depending on the physical treatments. The highest potency was found for the annealed-curdlan sample after it had been kept in 0.3M sodium hydroxide solution for 10 min, followed by neutralization prior to the assay. A similar observation was made for two branched (1→3)-β-D-glucans, HA-β-glucan and schizophyllan (Fig. 4). In particular, the potency for activation was remarkably enhanced by treatment of schizophyllan with sodium hydroxide solution (more than 100-fold; Fig. 4, a) or dimethyl sulfoxide (Fig. 4, b). Treatment of HA-β-glucan with either dimethyl sulfoxide or sodium hydroxide also resulted in a remarkable enhancement of Factor G activation (Figs. 4 c and d).

Host-mediated antitumor activity. — Examination of the antitumor activity of linear and branched (1→3)-β-D-glucans, as determined by the growth inhibition of tumors, indicated clearly that curdlan obtained by lyophilization of a dimethyl sulfoxide solution, HA-β-glucan, and schizophyllan were effective at a dose of 1 mg/kg, whereas curdlan powder and annealed curdlan were not effective (Table II). Curdlan powder became effective at a dose of 10 mg/kg, but laminaran remained ineffective at this concentration.

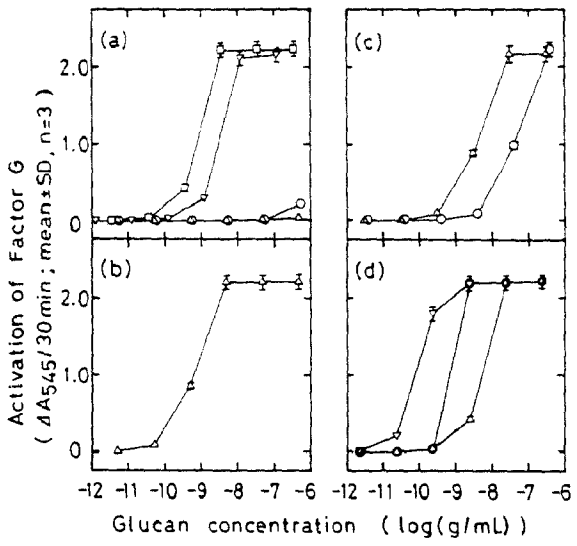

Fig. 4. Activation of coagulation Factor G from LAL by branched (1→3)-β-D-glucans. Panel a: Schizophyllan: (○) untreated commercial preparation; NaCl-treated sample (△); and NaOH-treated sample before (□) and after (▽) dialysis and lyophilization. Panel b: Schizophyllan dissolved in dimethyl sulfoxide solution (2 mg/mL), followed by dialysis against distilled water (overnight). Panel c: HA-β-glucan, lyophilized from aqueous solution (○) or dimethyl sulfoxide solution (△). Panel d: HA-β-glucan: Supernatant solution of aqueous suspension (10 mg/mL) before (○) and after treatment with 0.4M NaOH on ice cooling for 5 min, followed by neutralization with HCl (▽), or after treatment with 0.33M NaCl as a control (△). For the error bars, see the legend to Fig. 3.

Relationship between conformation and biological response. — On the basis of the foregoing n.m.r. data (Table I, Figs. 1 and 2), it is evident that the pronounced enhancement of the potency of activation of Factor G from LAL is related to the complete or partial formation of the single-helix conformation by treatment with dimethyl sulfoxide or sodium hydroxide solution. The single or triple helices are disrupted into random-coil form in an alkali concentration over 0.2M sodium hydroxide[34-36]. The single-helix form was subsequently recovered from the random-coil chains by neutralization[36]. This indicated a lack of aggregation of the single helical chains in aqueous solution, in contrast to the case of the curdlan obtained by lyophilization of a dimethyl sulfoxide solution. The potency of the activation of Factor G was very low for the latter (Fig. 3, a) and such a low activity is explained in terms of insufficient dispersion of the aggregated single helices into individual species as a stimulant for Factor G. Furthermore, there appeared no striking change between annealed curdlans obtained by rapid or slow cooling (Fig. 3, b). A previous ^{13}C-n.m.r. study had shown that the proportion of the triple helix formed is 100 and ~50% for the slowly and the rapidly cooled curdlan solution[23]. This may be again explained in terms of insufficient swelling, which resulted in a mixture of single chain and helix. By contrast, the polymer chains of the branched D-glucans are sufficiently swollen in aqueous media, and the high potency of activation of Factor G was clearly seen, even when the HA-β-glucan was obtained by lyophilization of a dimethyl sulfoxide solution (Fig. 4, c).

TABLE II

Antitumor activity of (1→3)-β-D-glucans in mice

Sample	*Physical state*	*Dose (mg/kg)*	*Inhibition ratio (%)*[a]	*Complete regression*
Curdlan	Annealed at 180°	10	56.8 (20.6)	1/6
		1	25.9 (23.7)	0/6
	Anhydrous powder	10	99.5 (0.5)[b]	5/6
		1	35.8 (21.3)	2/6
	Lyophilized from Me_2SO soln.	10	92.7 (6.6)[b]	3/6
		1	95.4 (4.0)[b]	4/6
HA-β-glucan	Lyophilized from aq. soln.	1	99.9 (0.07)[b]	5/6
Schizophyllan	Lyophilized from aq. soln.	1	99.9 (0.07)[b]	5/6
	NaOH-treated	1	100.0 (0)[b]	6/6
Laminaran	Lyophilized from aq. soln.	1	−44.8[c]	0/5

[a] ± S. E. in parentheses. [b] Significant difference from control $p < 0.001$ (Student's t test). [c] Control data different from the rest of the results shown here.

It appears that the potency of activation of Factor G from LAL (Figs. 3 and 4) parallels the antitumor activity (Table II), as far as conformations of glucans are concerned (Table I). Thus, laminaran, which is in a random coil in an aqueous medium, was ineffective for both the activation of Factor G (Fig. 3, c) and the antitumor activity (Table II). The single-helix conformation was more effective for the host-mediated antitumor activity of curdlan than was the triple-helix form (Table II). There was also a difference in response of schizophyllan for the two biological systems; no significant change in the antitumor activity was observed between a sample obtained by lyophilization of an aqueous solution (triple helix only) and a sodium hydroxide-treated sample (ratio of triple helix to single helix, 3:2; Table I). It is probable that a conformational change occuring between the single and triple helices during the long-time span of the assay (five weeks) may have obscured the selectivity of the single helix as an active conformation in the antitumor activity of the branched (1→3)-β-D-glucans. Such a plausible conformational change, however, may not occur within 18 h in the case of schizophyllan, as the same potency of activation of Factor G was retained during the course of dialysis of the dimethyl sulfoxide solution against distilled water (Fig. 4, b). The sparing solubility of the sample in aqueous medium, however, was generally not an obstacle for the *in vivo* assay of the antitumor activity, because of the probable involvement of several subsequent biological processes. Therefore, the aforementioned differential responses between the *in vivo* and *in vitro* assays can be accounted for by the ease of conformational change during the time required for the respective assay.

In conclusion, a very sensitive relationship between the potency for the activation of Factor G and the presence of the single-helix conformation in (1→3)-β-D-glucans, as shown by high-resolution, solid-state ^{13}C-n.m.r. technique, was demonstrated. In addition, an apparent parallelism in the conformation–biological response relationship between the activation of Factor G in LAL and the antitumor activity as a whole was observed.

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