INFLAMMATORY AND IMMUNOTOXICOLOGICAL ACTIVITIES OF (1_3)-β-GLUCANS

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Abstract

(1→3)-β-Glucans exhibit a variety of biological activities, and the significance of these activities is dependent on the structure of the β-glucans, such as solubility in water, molecular weight, degree of branching, and conformation. We have been analyzing the relationship between structure and activity of "gel-forming, high-molecular weight, soluble β-glucans," such as schizophyllan (SPG), SSG, OL-2, and grifolan (GRN). We have a tentative conclusion that the soluble β-glucans have almost no toxicity and that some of the activities, such as NO synthesis, vascular permeability, inflammatory cytokine synthesis, and limulus factor G activation, are significantly dependent on the conformation (single helix) of the β-glucans. In addition, only GRN produced significantly high concentration of TNF-α from macrophages in vitro. However, we exceptionally found that all the β-glucans tested showed lethal toxicity in mice when administered with indomethacin. Large number of mice died within a week from the β-glucan administration. Cytokine concentrations in sera of these mice were significantly increased by this treatment, suggesting that the lethal toxicity might be related to aberrance of the cytokine network.

Introduction

(1→3)-β-D-glucans are present in a variety of sources, including fungi, yeast, algae, bacteria, and higher plants, and in comparison with bacterial lipopolysaccharide (LPS), their structures also vary significantly depending on molecular weight, degree of branching, solubility in water, and ultrastructure (Stone and Clarke 1992). In addition to the structural variability, (1→3)-β-D-glucans show a variety of biological activities, such as protection of the host against cancer, microbes, and radiation. In contrast to these beneficial effects, a β-glucan containing particle, zymosan prepared from a yeast, *Saccharomyces cerevisiae*, is a well-established model substance showing acute inflammatory responses. Increasing incidence of fungal infections in relation to transplantation, chemo/physico-therapy of cancer patients, and immunosuppressing viral infection led us to clarify the contribution of β-glucan in the patho-physiology of the disease. Thus, studies on the structure-activity relationships of both soluble and particulate forms of (1→3)-β-D-glucan with the parameters of the host defense mechanisms have been worthwhile.

The β-glucans have been isolated and purified from fungi, including mushrooms, yeasts, and cultured mycelia. Two of the β-glucans, lentinan from *Lentinus edodes*, and sonifilan (SPG) from *Schizophyllum commune*, have been approved for clinical use in cancer patients in Japan. We had the opportunity to use SPG in addition to our own β-glucan preparations and compared precisely each of their biological activities. This paper summarizes the relationship between structure and immunopharma-/ immunotoxi-cological activity of soluble and particulate (1→3)-β-D-glucans, including conformation dependency.

Results

Structural Features of (1→3)-β-D-Glucan

The primary structure of the β-glucans from fungi was usually illustrated simply as a 3-linked β-D-glucopyranosyl residue (main chain) in the presence or absence of 6-linked β-D-mono-glucopyranosyl residue (side chain). The degree of branching (DB), ratio of the branch, is an important factor that specifies immunopharmacological activity. Previous studies strongly suggested that a branching ratio of less than 1/2 (one branch in every other main-chain unit) is important to exhibit significant antitumor activity against the solid transplantable tumor, sarcoma 180, in ICR mice. The structure-activity relationships of the other activities were, however, different as shown in Table 1 (See also review, Yadomae and Ohno 1996; Ohno et al., 1997). It is also noteworthy that 6-branched β-glucosyl residue is usually a monosaccharide branch. In contrast, yeast cell wall usually contains (1→6)-β-D-glucan chains in addition to (1→3)-β-D-glucan chains.

Results of X-ray crystallography and physical studies strongly suggested the structure as a straight rod in which three main chain moieties are located inside by interchain hydrogen bonding to form a “triple helix” structure. The side-chain moiety is localized outside the rod. However, it is not the only conformer of soluble (1→3)-β-D-glucans, which possess three kinds of conformers: the triple helix, single helix and random coil. The conversion of single to triple or triple to single helix conformers is mediated by several chemical- and physical-treatments, such as treatment with sodium hydroxide, urea, or dimethylsulfoxide or treatment above 135 °C (Adachi et al., 1990). The single helix conformer gradually changed to the triple helix because a triple helix conformer is usually more stable than the single helix conformer. However, not all the single helix conformers were changed to the triple helix and a certain proportion of the single helix segment remained in the treated preparation. The ratio of the single versus triple helix conformers could not be measured exactly but be estimated by a diagnostic test using limulus factor G that is specific for the single helix and the random coiled conformers but does not react with the triple helix (Nagi et al., 1993).

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Structure Activity Relationship

To examine the structure-activity relationship of (1→3)-β-D-glucan, we prepared several β-glucans, namely GRN, DB=1/3 from *Grifola frondosa*, SSG, DB=1/2 from *Sclerotinia sclerotiorum*, and OL-2, DB=2/3 from *Omphalia lapidescence*. In addition we used SPG (Sonifilan; used clinically in Japan), DB=1/3 from *Schizophyllum commune*, curdlan (CRD), DB=0/0 from *Alcaligenes faecalis*, and laminaran. SPG was once treated with 0.5N NaOH to prepare the single helix conformer (SPG-OH). As the particulate β-glucan preparation, we used zymosan A (ZYM, not a pure β-glucan preparation) and zymocel (ZYC). The activities tested in our laboratory were summarized in Table 1. From these data we have tentatively concluded that the structure-activity relationship of each activity is not simple. For example, the antitumor activity to the solid tumor was strong in the cases of β-glucans having moderate percentage of branching ratio. Priming effect on LPS triggered TNF-α synthesis was also strong in the single helix conformer. Of quite importance, activity of SPG-OH was significantly different from that of SPG. Many of the activities, release of Evans blue, limulus reactivity, cytokines syntheses *in vitro*, could not be induced by SPG. In addition, a linear β-glucan, CRD, showed significantly lower activity than branched β-glucans in many experimental systems. These facts strongly suggested an invaluable contribution of sidechain moieties for inducing the biological activity.

Nitric Oxide and Inflammatory Cytokine Synthesis by Macrophage

Fungal β-glucans have abilities to induce NO synthesis by macrophages *in vivo*, and the intensity of NO synthesis significantly varied dependent on the structure of β-glucans (Table 2). NO synthesis could not be induced by SPG. In the model experiment using recombinant cytokines, a minimum concentration of the cytokines for NO induction was about 20 ng/ml in the presence of IFN-γ under the experimental conditions (Table 3). IFN-γ and CSF synthesis were enhanced by various β-glucans including both single and triple helix conformers (Tables 4 and 5). The priming effect for the LPS triggered TNF synthesis was also enhanced, but weaker in the case of SPG (Ohno et al., 1995). To determine the mechanism of β-glucan-mediated NO production, peritoneal macrophages (PMs) were stimulated *in vitro* by β-glucans in the presence of IFN-γ. Some β-glucans induced NO synthesis *in vivo* but not *in vitro* (OL-2, ZYM, ZYC) (Table 2). Of β-glucans tested, only SPG-OH and GRN produced high concentrations of IL-1 and IL-6 in the culture supernatants (Table 6 and 7). SSG also induced NO synthesis *in vitro*, but concentrations of inflammatory cytokines were not prominent even in the presence of IFN-γ. These findings suggested that NO productivity of β-glucans *in vivo* is regulated by several mechanisms.

Lethal Toxicity Induced by Combination Use of β-glucan and Indomethacin

As shown in the introduction, it is no doubt that particulate β-glucans do and soluble β-glucans do not show apparent toxicity that includes severe inflammation. However, during analysis of the molecular mechanism of the soluble β-glucan mediated immuno-pharmacological activity, we have found a strong lethal toxicity of soluble β-glucans when administered with indomethacin (Table 8). Mice were died gradually nearly a week. Following analysis of patho-physiological parameters, we found significantly high concentrations of IL-6, CSF (Table 9), IFN-γ, NO, and acute phase proteins in sera. From these data it is most possible that the combination use of β-glucan and indomethacin induced a pathosis, similar to the systemic inflammatory response syndrome (SIRS).

Conclusion

Soluble β-glucans, Lentinan and SPG, have been used clinically in cancer patients with beneficial effects. We have shown that (1→3)-β-D-glucans exhibit a variety of biological and immuno-pharmacological activities, and the significance of these activities is dependent on the structure of the glucans including solubility in water, molecular weight, degree of branching, and conformation (Table 1). It is tentatively concluded that the biological activity of the soluble glucans is moderate compared with the particulate glucans. Thus they showed beneficial and pharmacologically useful effects with almost no toxicity. Only soluble β-glucans have been applied clinically.

Incidence of allergic broncho-pulmonary diseases is increasing. At least a part of the disease would be associated with the fungal infection. β-Glucan is a major component of the fungal cell wall, thus, it is plausible that the β-glucan contributes to the pathosis of the disease. It is strongly supported by the fact that the particulate β-glucan preparations, ZYM and ZYC, have been well known to induce strong inflammatory responses. In addition, we have shown in this paper that the concentration of the cytokines induced by β-glucans were significantly modulated by indomethacin and induced die. These data strongly suggested that soluble β-glucans have immunomodulating as well as immunotoxicological activities.

Summary

(1→3)-β-Glucans exhibit a variety of biological activities, and the significance of these activities is dependent on the structure of the β-glucans, such as solubility in water, molecular weight, degree of branching, and conformation. We have a tentative conclusion that the soluble β-glucans have almost no toxicity. However, the combination use of β-glucan and indomethacin induced a pathosis, similar to the systemic inflammatory response syndrome (SIRS). These data strongly suggested that β-glucans have immuno-modulating as well as immuno-toxicological activities.

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Table 2. NO Synthesis by β-Glucans in Mice

	NO (μM)	
	in vitro (+ IFN-γ)	*in vivo*[c)]
saline	4.4±0.9	3.1±0.7
SPG	2.0±0.2	5.8±1.5
SPG-OH	17.6±2.0***	35.5±7.5***
SSG	11.5±6.7**	31.5±2.2***
GRN	15.0±2.7***	37.6±5.3***
OL-2	5.1±0.9	64.2±3.4***
CRD	N.D.	3.0±0.5
LAM	3.8±0.6	N.D.
ZYM (i.p.)	5.0±0.5*	20.5±3.5**
(i.v.)	N.D.	N.D.
LPS	N.D.	N.D.

Values represent mean ± S.D. Significant difference from control, * p<0.05, ** p<0.01, *** p<0.001.

Table 3. Effects of Cytokines on NO Synthesis of PMs *in vitro*.

		+medium	+IFN-γ
Medium		2.8±0.6(uM)	4.4±0.9
TNF-α	10 (ng/ml)	2.9±0.2**	7.0±1.2***
	25	4.8±1.2**	17.5±6.6**
GM-CSF	10	3.5±0.7*	4.3±0.1
	25	4.0±0.9**	14.2±6.5**
IL-1α	10	3.3±0.3*	4.2±0.6
	25	4.0±0.9**	13.5±6.7**
IL-6	10	5.2±1.0***	6.9±1.6**
	25	5.7±1.6***	20.1±8.1**
LPS	10 (ng/ml)	4.6±1.3***	5.3±0.8***
IFN-γ	1 (U/ml)	2.4±0.3	?
	10	4.4±0.9	?
	100	9.6±1.3	?

Proteose peptone-elicited PMs were incubated with cytokines (10, 25ng/ml) or LPS (10ng/ml) in the presence or absence of IFN-γ (10U/ml) for 24h. NO concentration was determined by Griess reagent. Values represent mean ± S.D. Significant difference from control (medium), * p<0.05, ** p<0.01, *** p<0.001.

Table 4. IFNγ Synthesis by β-Glucans in Mice

	IFN-γ *in vivo*	
	sera	spleen
saline	2.5±1.9	4.7±1.3
SPG	8.2±4.4**	19.1±7.7***
SPG-OH	9.2±4.9***	23.2±10.7***
SSG	7.5±4.5***	16.2±9.4***
GRN	5.6±1.1***	16.2±4.8***
OL-2	9.4±4.7**	25.5±4.6***
CRD	5.2±1.2	9.9±6.5**
LAM	4.8±0.9**	5.9±3.3
ZYM (i.p.)	2.7±1.8	12.8±8.5**
(i.v.)	2.8±1.5	17.9±15.0**
LPS	3.4±0.6	21.9±18.5*

[a)]Values represent mean ± S.D. Spleen cells were cultured in vitro for 1 day to collect supernatant. Significant difference from control, * p<0.05, ** p<0.01, *** p<0.001.

Table 5. CSA in Sera and in Ascites of ICR Mice by Intraperitoneal Administration of Various (1_3)-Glucans.

	number of colonies (mean ± SD)		
	serum (3h)	serum (day 7)	ascites (3h)
Nil	5.3 ±3.8	9.5±2.9	27.0±3.3
OL-2	7.8 ±1.3	30.0±6.7**	N.D.
SSG	56.8 ±16.4***	38.9±9.7***	160.0±18.9***
GRN	45.1 ±16.5***	30.3±9.3*	23.5±6.1
SPG	28.8 ±4.4***	32.0±5.5***	23.5±1.5
SPGOH	26.1±9.2***	35.3±11.5***	51.3±11.5*
ZYM	13.0±4.7*	7.8±7.3	53.8±12.4*
CRD	16.3±3.3**	13.5±1.1*	N.D.
PAR	2.0±1.2	4.0±1.6**	N.D.

Each 250 μg of glucan was i.p. administered on day 0 (0 h). * p<0.05, ** p<0.01, *** p<0.001

Table 6. Effects of β-Glucans on cell bound IL-1 Production of PMs *in vitro*.

	IL-1α (cell lysate)	
	+ medium	+ IFN-γ
Medium	2.7 ± 0.4	4.6 ± 0.4
SPG	5.0 ± 0.8**	8.8 ± 1.3**
SPG-OH	66.1 ± 10.3***	218.6 ± 64.7**
SSG	33.9 ± 20.8*	44.9 ± 13.6**
GRN	873.8 ± 274.4**	1653.9 ± 334.5**
OL-2	8.3 ± 0.1***	11.6 ± 2.3**
LAM	0	2.0 ± 0.2
ZYM	123.5 ± 10.8*	257.8 ± 5.1**
ZYC	7.2 ± 0.4**	9.0 ± 0.2**

Proteose peptone-induced PMs were incubated with β-glucans (100μg/ml) in the presence or absence of IFN-γ (10U/ml) for 24h. Cytokine levels in the culture supernatant and cell lysates were determined by ELISA. Values represent mean±S.D. Significant difference from control (medium), * p<0.05, ** p<0.01, *** p<0.001.

Table 7. Effects of β-Glucans on IL-6 Production of PMs *in vitro*

	IL-6 (pg/ml)	
	+ medium	+ IFN-γ
Medium	36.7±12	130.0±33.9
SPG	78.0±28.5***	213.7±75.0***
SPG-OH	12325.2±1553.4***	22335.6±4831.4***
SSG	194.3±118.2***	412.0±223.5***
GRN	13354.1±1611.2***	18607.9±3466.6***
OL-2	573.2±78.0***	1448.4±76.9***
LAM	59.5±7.5***	100.7±17
ZYM	3173.7±440.1***	3475.17±195.3***
ZYC	939.2±123.0***	1040.5±211.7***

Proteose peptone-induced PMs were incubated with β-glucans (100μg/ml) in the presence or absence of IFN-γ (10U/ml) for 24h. Cytokine levels in the culture supernatant and cell lysates were determined by ELISA. Values represent mean±S.D. Significant difference from control (medium), * p<0.05, ** p<0.01, *** p<0.001.

Table 8. Effect of Various β-Glucans on Mortality of INDO-administered mice.

	Treatment Glucan	Life kSpan (days, mean ± SD)	No. of mice dead/total on day 14
Expt			
1		8.8 ± 5.5	5/8
	SPG	3.8 ± 1.0*	8/8
2		12.0 ± 3.3	3/10
	SSG	4.0 ± 3.6***	9/10
	GRN	5.0 ± 4.7***	8/10
	SPG-OH	6.2 ± 4.6**	8/10
	ZYM	9.7 ± 4.2	6/10
3		14.0 ± 0	0/10
	OL-2	7.4 ± 4.4***	8/10
	CRD	14.0 ± 0	0/10
	LAM	13.2 ± 2.4	1/10
	ZYC	11.9 ± 4.2	2/10

IND (5 mg/kg) was p.o. administered to mice and β-glucan (250 μg/mouse) was i.p. administered 1 h later on day 0. INDO was p.o. administered once a day from day 1 to day 14 and mortality was monitored. The significance was evaluated by Student's *t*-test against the control group. * $p<0.05$ ** $p<0.01$ *** $p<0.001$

Table 9. Enhancement of Cytokine Productivity in Mice by SSG/INDO Treatment.

	IL-6 in serum (pg/ml)	CSF in serum (CFU)
Nil	8.1±16.8	0.1±0.4
SSG	30.9±40.2	8.3±9.1*
INDO	23.3±38.0	1.4±1.8*
SSG+INDO	328.6±142.2***	45.4±18.6***

SSG (250μg/mouse) were administered 3 times every other day from day 0. INDO were administered on day 7 by p.o. Sera were obtained on day 8. *, p<0.05; ***, p<0.001

Table 1. Immunopharmacological Activities of Soluble and Particulate β-Glucans.

	LAM	CRD	SPG	SPG-OH	GRN	SSG	OL-2	Particle
Source	*Laminaria digitata*	*Alcaligenes faecalis*	*Schizophyllum commune*	*Schizophyllum commune*	*Grifola frondosa*	*Sclerotinia sclerotiorum*	*Ompharia lapidescence*	*Saccharomyces cerevisiae*
Branching ratio	DB=0/6	DB=0/6	DB=2/6	DB=2/6	DB=2/6	DB=3/6	DB=4/6	DB=0/6
Average Mol. Wt.	5×10^4	DPn 450	4.5×10^5	1.5×10^5	$>10^6$	$>10^6$	2.5×10^5	
(solvent)			(H_2O)	(NaOH)	(NaOH)	(NaOH)	(NaOH)	
Conformation	r	s + t	t	s + t	s + t	s + t	s + t	s + t + r
% of triple helix (by limulus G test)			0	89	93	1	8	
<<< in vivo >>>								
Blood clearance			slow	fast				
Antitumor activity (solid)	×	△	◎	◎	◎	◎	△	○Zym ◎Zyc
Antitumor activity (ascites)	×		×		◎	◎	◎	
Colony stimulating factor production (ascites)		×	○	◎	○	◎	○	○
Colony stimulating factor production (serum)			○	○	○	○	○	
Enhance colony forming unit in bone marrow			○	○	○	○		○
Recovery from cyclophosphamide induced leukopenia			◎	◎	◎	◎		◎(Zyc)
NO synthesis of PEC		×	×	◎	◎	◎	◎	○Zym ◎Zyc
Enhance H_2O_2 synthesis of PEC			△	○	◎	◎	◎	◎
IFN-γ synthesis (serum)	△	×	○	○	○	○	○	×
IFN-γ synthesis (spleen)	×	○	◎	◎	◎	◎	◎	◎
Priming effect on LPS triggered TNF-α_synthesis	×	×	△	○	○	◎	◎	○
Synthesis of membrane TNF-α					○	○		
Extravascular release of Evans blue	△	○	×	△	○	○	○	◎
Lethal toxicity (+ Indomethacin)	×	×	◎	◎	◎	◎	◎	△
<<< in vitro >>>								
Limulus reactivity	○	◎	×	○	○	○	○	◎
Silkworm larvae reactivity	◎	◎	○	○	○	○	○	◎
Plasma clotting		fast	slow	fast	fast	fast	slow	
Protein binding			×	○	○			◎
Complement activation (APC)		◎	△	△	○	△	◎	◎
Synthesis of membrane IL-1α by Macrophage	×		△	○	◎	○	△	△
Synthesis of IL-6 by Macrophage	△		△	◎	△	△	◎	○
Synthesis of TNF-α by Macrophage	×	×	×	○(+ IFN-γ)	○	×	×	◎Zym ○Zyc
Synthesis of chemotactic factor			×		○	×		○
Synthesis of NO by Macrophage (+IFN-γ)	×	△	×	○	○	○	×	△Zym △Zyc
H_2O_2 synthesis of macrophage			×	×	×	×	×	○Zym ◎Zyc
Arachidonic acid release from Macrophage (JA-4)		×	×		×	×		◎
Antagonist to β-GR (H_2O_2 synthesis in vitro)			◎	○	○	○	○	(agonist)

LAM; laminaran, CRD; curdlan, SPG; sonifilan, SPG-OH; sodium hydroxide treated SPG, GRN; grifolan, DPn; degree of polymerization, DB; degree of branching, s; single helix, t; triple helix, r; random coil, PEC; peritoneal exudate cell, APC; alternative pathway , GR; glucan receptor, ×; negative, △; weak, ○; strong, ◎; very strong, blank well; not determined.