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Antitumor and Immunomodulating Activities of a β-Glucan Obtained from Liquid-Cultured *Grifola frondosa*

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The effects of the β-1,3-glucan, LELFD, obtained from liquid-cultured mycelium of *Grifola frondosa*, on the growth of syngeneic tumors and immune responses in mice were examined. In Meth A or IMC solid tumor systems, LELFD administered intraperitoneally (i.p.) or intralesionally (i.l.) exhibited significant antitumor effects. However, the growth of L1210 and P388 leukemias was unaffected by the injection of LELFD.

The injection of LELFD i.p. enhanced the activities of natural killer cells and macrophages in mice. LELFD also enhanced the antibody response when it was injected i.p. with sheep red blood cells into mice. Furthermore, it was found that LELFD could activate the alternative complement pathway.

Keywords *Grifola frondosa*; LELFD; 6-branched β-1,3-glucan; antitumor activity; adjuvant; natural killer cell; macrophage

Introduction

In has been shown that fruit bodies and culture filtrates of various fungi contain antitumor polysaccharides of which the structure is mainly a 6-branched β-1,3-glucan.[1-5] Lentinan from *Lentinus edodes* and schizophyllan from *Schizophyllum commune* have been employed clinically as anticancer drugs.

Grifola frondosa is a fungus belonging to the Polyporaceae (Basidiomycetes) and the fruiting bodies have been used as an edible mushroom. We reported previously that incubation of the mycelium of *G. frondosa* in a buffer containing glucose and citric acid produced large amounts of polysaccharide fraction which contained 6-branched β-1,3-glucan.[6] The polysaccharide fraction named LELFD showed a significant antitumor activity in allogeneic tumor systems.[6] The method of preparation of LELFD is quite simple and milder than the procedures used to obtain other antitumor glucans such as hot water extraction (lentinan[1]) and PS-K[7]) or alkali extraction (grifolan NMF-5N[5]). Furthermore, the resultant preparation contained only a small amount of materials originating from the broth. We consider therefore that the preparation method of LELFD represents an excellent technique for obtaining antitumor polysaccharide, and LELFD is worthy of study to determine whether it exhibits similar antitumor and immunomodulating activities to other antitumor polysaccharides.

In the present investigation, we examined both the antitumor activity in syngeneic murine-tumor systems and the immunomodulating activities in some immunological functions of LELFD.

Materials and Methods

Mice Male 6- to 8-week-old BALB/c, CDF_1, DBA/2, C3H/HeN, and ICR mice were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka. The mice were placed under specific pathogen free conditions.

Tumors Meth A fibrosarcoma cells and IMC carcinoma cells were maintained in the peritoneal cavities of BALB/c and CDF_1 mice, respectively. L1210 leukemia cells and P388 leukemia cells were also maintained in the ascites form by serial intraperitoneal passage through DBA/2 mice. YAC-1 lymphoma cells were subcultured in RPMI 1640 medium (Nissui Seiyaku Co., Tokyo) containing 10% heat-inactivated fetal calf serum (FCS, Grand Island Biological Co., Grand Island, NY), 5 mM *N*-hydroxyethylpiperazine-*N'*-2-ethansulfonate (HEPES), 100 U/ml of penicillin and 100 μg/ml of streptomycin (termed "complete medium" hereafter).

Preparation of LELFD The polysaccharide fraction was prepared from culture filtrate of *Grifola frondosa* as described previously.[6] Briefly, *G. frondosa* var. Tokachiana was grown in 100 ml of medium containing glucose (2.0%), polypeptone (0.6%), cane sugar (2.0%) and soybean oil (0.1%), pH 4.5, with reciprocal shaking at 25°C for 14 d. The mycelium was separated from the filtrate by filtration, and lyophilized. The lyophilized mycelium (13 g) was suspended in a buffer containing glucose (5.0%) and citric acid, pH 4.5. The suspension was incubated at 25°C for 3 d. The supernatant was separated by centrifugation. These procedures were repeated 2 to 3 times. The combined supernatant was dialyzed against water and non-dialyzable fraction was precipitated with ethanol (LELFD, 6.3 g).

Agents Lentinan was kindly supplied by Yamanouchi Seiyaku Co. (Tokyo). Picibanil (OK-432) was kindly supplied by Chugai Seiyaku Co. (Tokyo). Lipopolysaccharide (LPS) was purchased from Difco Laboratories (Detroit, MI). Concanavalin A (Con A) was purchased from Miles-Yeda (Kankakee, IL).

Evaluation of Antitumor Activity Tumor cells were harvested from ascites fluid, washed with Hanks' balanced salt solution (HBSS, Nissui), and suspended in HBSS. Meth A fibrosarcoma cells (5×10^5), L1210 leukemia cells (1×10^5), or P388 leukemia cells (1×10^6) were inoculated subcutaneously (s.c.) into the right groin of corresponding mice as described elsewhere. IMC carcinoma cells (5×10^5) were inoculated intradermally (i.d.) into the right flank of CDF_1 mice. Test samples dissolved in saline or saline alone were injected i.p. or i.l. several times from 1 d after tumor implantation. For evaluation of the antitumor activities against Meth A and IMC tumor cells, these mice were killed on day 35, and the tumors were weighed. The antitumor activity was assessed from the percent inhibition which was calculated as: [1-(average tumor weight of the treated group/average tumor weight of the control group)] × 100(%). The antitumor effects against L1210 and P388 tumor cells were evaluated in terms of the $T/C(\%)$ of the survival time, where T=survival (d) of the tested group and C=survival (d) of the control group.

Assay of Acid Phosphatase Activity in Macrophages Mice were injected i.p. with test samples. Four days later, peritoneal exudate cells (PEC) were collected from the mice by washing the peritoneal cavity twice with 5 ml of HBSS containing heparin (5 U/ml). The cells were washed twice and resuspended in RPMI 1640 medium. The total cell number was counted with a hemocytometer, and the cells were differentiated using a Diff-Quik Stain Kit (Kokusai Shiyaku Co., Hyogo). A 1.0 ml portion of the cell suspension containing 5×10^5 macrophages was placed in a flat-bottomed 24-well tissue culture plate (Sumitomo Bakelite Co., Tokyo). After incubation for 2 h at 37°C in a CO_2 incubator, nonadherent cells were removed by washing twice with RPMI 1640 medium, and 0.1 ml of 0.1% Triton X-100 was added to the well. Then, 0.5 ml of *p*-nitrophenyl phosphate (PNP) solution as a substrate and 0.4 ml of 0.1 M citrate buffer (pH 5.0) were added to the well. Following incubation for 1 h at 37°C, 1 ml of 0.2 M borate buffer (pH 9.8) was added to the reaction mixture, and the optical density at 405 nm was measured.

Assay for Natural Killer (NK) Cell Activity The cytolytic activities of spleen cells taken from sample-injected mice against YAC-1 lymphoma

cells were assessed by measuring the ^{51}Cr release. YAC-1 cells were labeled by incubation at 37 °C for 1 h with $Na_2{}^{51}CrO_4$, washed and then incubated again at 0 °C for 1 h. The cells suspended in complete medium were mixed with effector cells in a 96-well round-bottomed microtiter plate (Sumitomo; total, 0.2 ml). After incubation for 5 h at 37 °C in a CO_2-incubator, the supernatant (0.1 ml) from individual wells was collected, and the radioactivity was counted in a gamma scintillation counter.

Mitogenicity Assay Mitogenic activity was assessed by the method described previously.[8]

Plaque-Forming Cell (PFC) Assay The PFC response to SRBC was determined by the technique of Cunningham and Szenberg.[9]

Activation of Alternative Complement Pathway Freshly prepared samples of human whole serum provided sources of complement. For assay, samples were dissolved in gelatin veronal buffer (GVB) containing 0.05 M ethylene glycol bis-amino tetraacetate (EGTA) and 0.01 M magnesium (EGTA–GVB·Mg^{2+}). Then, 0.1 ml of the sample was incubated with the complement solution (0.4 ml) at 37 °C for 1 h, and diluted with 0.01 M EGTA–GVB·Mg^{2+}. The solution (0.8 ml) was mixed with 0.2 ml of rabbit red blood cells (RBC, 5×10^7/ml), and incubated at 37 °C for 1 h. A 2.0 ml portion of GVB containing 0.01 M ethylenediamine tetraacetate (EDTA) (EDTA–GVB) was added to the solution to block further complement activation. After centrifugation, the optical density of the supernatant was read at 414 nm.

Statistics The significance of differences between means was determined by the use of Student's t test.

Results and Discussion

Examination of Antitumor Activity of LELFD The antitumor effects of LELFD were examined in several syngeneic murine tumor systems including Meth A fibrosarcoma-BALB/c, IMC carcinoma-CDF_1, L1210 leukemia-DBA/2 or -CDF_1, and P388 leukemia-CDF_1. As shown in Table I, LELFD injected i.p. or i.l. exhibited significant antitumor effects against both Meth A fibrosarcoma and IMC carcinoma, and the respective inhibition ratios were between 64.5 and 85.3. However, i.p. or i.l. injection of LELFD into mice implanted with L1210 or P388 leukemia did not prolong the survival times of the mice.

Examination of Immunomodulating Activities of LELFD The effect of LELFD on the macrophage function was first evaluated as follows. The acid phosphatase activity of peritoneal macrophages taken from mice which had been administered with LELFD was compared with those from normal or OK-432-injected mice. As shown in Table II, i.p. injection of LELFD (75 or 150 μg) enhanced the activity of acid phosphatase, although the enhancing effect was lower than that induced by OK-432.

The effect of LELFD on the NK activity of spleen cells was examined by using NK cell-sensitive target tumor cells (YAC-1 lymphoma). Mice (C3H/HeN) were administered with 75 μg of LELFD i.p. on the day before cytolysis assay. As shown in Table III, the administration of LELFD augmented the cytolytic activity towards the YAC-1 cells.

The adjuvant effect of LELFD on the antibody response to sheep red blood cells (SRBC) was evaluated. Mice (ICR) were injected with LELFD i.p. and simultaneously with SRBC (1×10^7, i.p.). The anti-SRBC PFC responses on day 4 are shown in Table IV. The administration of LELFD significantly increased the number of PFC to SRBC, although a low dose of LELFD (30 μg) did not exhibit such an effect. The adjuvant effect of 100 μg of LELFD was particularly high and the stimulating index was 9.76.

The mitogenic activity of LELFD was also investigated in *in vitro* cultures of spleen cells from BALB/c mice.

TABLE I. Antitumor Effects of LELFD in Several Syngeneic Tumor Systems

Tumor	Sample (μg)	Treatment schedule		Route	No. of mice	Tumor weight[a] or survival days[b]	Inhibition ratio[c] or ILS (%)[d]	p[e]
		d	Times					
Meth A	LELFD 75	1, 3, 5, 7, 9	5	i.l.	5	0.65±0.69[a]	79.4	$p<0.02$
	LELFD 150	1, 3, 5, 7, 9	5	i.l.	5	1.13±0.65	64.5	$p<0.05$
	Saline	1, 3, 5, 7, 9	5	i.l.	5	3.17±1.62	—	
Meth A	LELFD 50	11, 13, 15, 17, 19	5	i.p.	5	0.46±0.20[a]	85.3	$p<0.05$
	LELFD 100	11, 13, 15, 17, 19	5	i.p.	7	1.52±1.01	51.3	
	Saline	11, 13, 15, 17, 19	5	i.p.	6	3.12±2.02	—	
IMC	LELFD 75	1, 3, 5, 7, 9	5	i.p.	10	2.27±3.10[a]	74.5	$p<0.001$
	LELFD 150	1, 3, 5, 7, 9	5	i.p.	10	2.96±2.64	66.9	$p<0.001$
	Saline	1, 3, 5, 7, 9	5	i.p.	12	8.93±3.43	—	
L1210[f]	LELFD 75	1—10	10	i.l.	8	11.50±0.76[b]	102.5	
	LELFD 150	1—10	10	i.l.	8	11.25±0.71	100.3	
	Lentinan 25	1—10	10	i.l.	8	11.88±1.13	105.9	
	Lentinan 50	1—10	10	i.l.	8	12.00±1.07	107.0	
	Lentinan 100	1—10	10	i.l.	8	11.00±0.93	98.0	
	Saline	1—10	10	i.l.	9	11.22±1.09	—	
L1210[g]	LELFD 100	1—10	10	i.p.	7	7.86±0.90[b]	99.5	
	LELFD 100	1—10	10	i.p.	7	8.14±0.69	103.0	
	Lentinan 25	1—10	10	i.p.	7	8.14±0.69	103.0	
	Lentinan 50	1—10	10	i.p.	7	8.14±0.69	103.0	
	Saline	1—10	10	i.p.	10	7.90±0.32	—	
P388	LELFD 75	1—10	10	i.l.	8	10.50±0.54[b]	101.6	
	LELFD 150	1—10	10	i.l.	9	10.33±0.71	100.0	
	Lentinan 25	1—10	10	i.l.	8	10.00±0.76	96.8	
	Lentinan 50	1—10	10	i.l.	8	10.13±0.84	98.1	
	Lentinan 100	1—10	10	i.l.	8	10.38±0.52	100.5	
	Saline	1—10	10	i.l.	9	10.33±0.87	—	

a) Arithmetic mean weight of tumor ± standard deviation. *b*) Arithmetic mean survival days of mice ± standard deviation. *c*) Results for Meth A fibrosarcoma and IMC carcinoma. *d*) Results for L1210 and P388 leukemias. *e*) The significance (p value) of difference between the control and treated groups was evaluated according to Student's t test. $p<0.05$ was taken as the criterion for a significant difference. *f*) L1210 leukemia cells were inoculated into DBA/2 mice. *g*) L1210 leukemia cells were inoculated into CDF_1 mice.

Table II. Effect of LELFD on Acid Phosphatase Activity in Mouse Peritoneal Macrophages

Sample[a]	Dose (μg)	Enzyme activity[b]	p[c]
LELFD	75	9.05±1.18	$p<0.01$
LELFD	150	10.03±2.05	$p<0.05$
OK-432	1KE	23.05±1.35	$p<0.001$
Saline		5.62±0.45	—

a) Each sample was administered i.p. into CDF_1 mice. *b*) *p*-Nitrophenol (nmol) per 5×10^5 macrophages per 60 min. *c*) See footnote *e*) of Table I.

Table III. Effect of LELFD on NK Cell Activity in Mice

	E/T ratio	Cytolysis (%, mean±S.D.)[a]	p[b]
LELFD	50/1	21.8±0.9	$p<0.001$
LELFD	100/1	22.6±1.6	$p<0.01$
Saline	50/1	11.9±2.0	—
Saline	100/1	16.4±1.3	—

a) The percentage of cytolysis was calculated from the following formula: cytolysis (%)=[{experimental release (cpm)−spontaneous release (cpm)}/{maximum release (cpm)−spontaneous release (cpm)}]×100. The maximum release was determined as the release of radioactivity into the supernatant fluid after addition of 1 N HCl to the labeled tumor cells. *b*) See footnote *e*) of Table I.

Table IV. Adjuvant Effect of LELFD on Antibody Response to SRBC

LELFD (μg)	Anti-SRBC PFC±S.E./spleen[a]	S.I.[b]	p[c]
30	978± 342	0.66	
100	14440±3961	9.76	$p<0.05$
300	5778±1311	3.90	$p<0.05$
Nil (saline)	1480± 194	1.00	

a) Mean anti-SRBC PFC number of 5 mice±standard error. *b*) Stimulation index. *c*) See footnote *e*) of Table I.

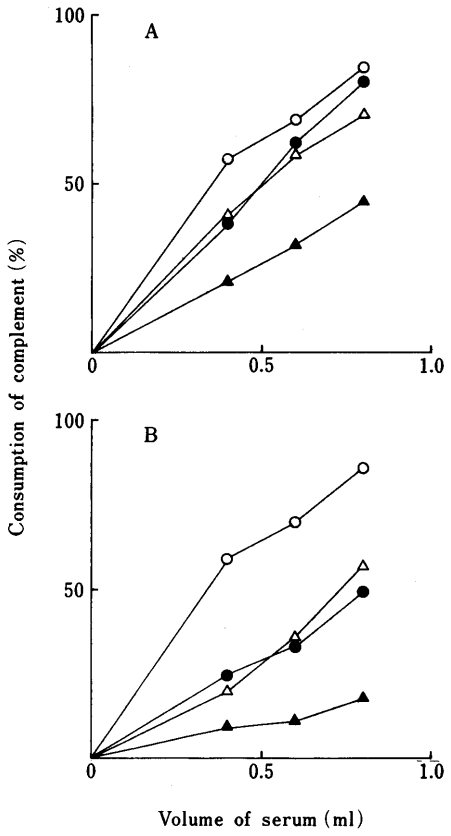

Fig. 1. Activation of the Alternative Complement Pathway by LELFD

A: —○—, nil; —●—, LELFD 0.6 mg/ml; —△—, LELFD 1.3 mg/ml; —▲—, LELFD 2.5 mg/ml. B: —○—, nil; —●—, LELFD 2.5 mg/ml; —△—, PS-K 2.5 mg/ml; —▲—, OK-432 0.6 mg/ml.

However, the addition of LELFD to the culture did not give rise to any mitogenic activity (data not shown).

The ability of LELFD to trigger the alternative pathway was compared with that of other immunomodulators (OK-432 and PS-K) using unsensitized rabbit RBC and human whole serum as a complement. As shown in Fig. 1, preincubation of the serum with 1.3 or 2.5 mg/ml of LELFD decreased the residual hemolytic activity. These results indicate that LELFD can activate an alternative complement pathway, although the ability seems to be lower than that of OK-432.

As described above, LELFD demonstrated significant antitumor activities against Meth A fibrosarcoma and IMC carcinoma. However, LELFD as well as lentinan did not show an antitumor effect against L1210 or P388 leukemia. The ineffectiveness of LELFD on leukemia may be due to the fast growing properties of these tumors.

Injection of LELFD into mice enhanced the lysozomal enzyme (acid phosphatase) activity of peritoneal macrophages. Schorlemmer *et al.*[10] described the importance of activated complement components in macrophage activation, leading to the release of lysozomal enzymes. LELFD was shown to possess an activation activity of the alternative complement pathway. This finding suggests that the activation of macrophages by LELFD may be due activation of complement. On the other hand, we and other laboratories have reported previously that several antitumor β-1,3-glucans could induce cytolytic or cytostatic macrophages against tumor cells.[11-14] It is possible therefore that macrophages may participate in the antitumor activity.

LELFD augmented the NK activity in the spleen when administered i.p. into mice. Considerable attention has been focused on NK cells which play an important role in the *in vivo* resistance against tumor development.[15,16] The NK activity enhancement by LELFD may also be important in the antitumor activity.

LELFD revealed an adjuvant activity on the antibody production in response to SRBC *in vivo*. However, it is considered that the activity was not due to a direct action on B cells because LELFD failed to show any mitogenic activity *in vitro*.

In the present study, the effects of a limited dose range of LELFD on the growth of tumors and immune responses in mice were investigated. It was impossible therefore to establish an optimal dose in each assay system. However, almost equal levels of activities were observed at LELFD doses of 75—150 μg/mouse, in most of the assays tested. These findings suggest that the optimal dose of LELFD for these activities is at approximately these doses.

LELFD revealed no direct cytotoxicity on tumor cells (data not shown). Taken together, this and the above described results suggest that the antitumor mechanism of LELFD may be host-mediated, and the immunopotentiating activities (particularly on macrophages and/or NK

cells) may be important in the antitumor activity.

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