Modulation of Antitumor Activity of Grifolan by Subsequent Administration of (1→3)- β -D-Glucanase *in Vivo*

Isao NONO, Naohito OHNO,* Masumi OHSAWA, Shozo OIKAWA** and Toshiro YADOMAE*

Laboratory of Immunopharmacology of Microbial Products, Tokyo College of Pharmacy,* Horinouchi, Hachioji, Tokyo, 192-03, Japan and Nippon Beet Sugar Mfg., Co., Ltd.,** Chuo-ku, Tokyo, 104, Japan

(Received March 24, 1989)

The initiation process of effector mechanism of the antitumor activity of (1→3)- β -D-glucans has been examined in detail by applying treatment of mice with (1→3)- β -D-glucanases, kitalase (KIT) and zymolyase (ZYM), *via* i.p. route after i.p. administration of (1→3)- β -D-glucans, grifolan (GRN) and CM-curdlan (CM-CUD). A significant decrease of antitumor activity caused by GRN was observed by treatment with KIT within 48 h, and that caused by GRN administered *via* i.v. route was also reduced by the i.v. injection of KIT, although it was less effective than i.p. treatment. Pretreatment of mice with thioglycollate reduced the period of time to abrogate antitumor activity by the sequential treatment of KIT. Similar results were also observed on ZYM treatment after i.p. treatment of CM-CUD. These facts suggest that the period of manifestation of the antitumor activity by (1→3)- β -D-glucans must be longer than a few days.

Keywords — (1→3)- β -D-glucan; grifolan; antitumor activity; (1→3)- β -D-glucanase

Introduction

Antitumor (1→3)- β -D-glucans have recently been used clinically for cancer immunotherapy.^{1,2)} The mechanisms of antitumor activity of (1→3)- β -D-glucan have demonstrated to activate the host immune system, and many experimental evidences including activation of macrophages,³⁾ killer T cells,^{4,5)} and natural killer cells,⁶⁾ and release of cytokines^{7,8)} seem to support this concept. Chemical modifications of these glucans have given evidences to demonstrate structure-function relationship, such as substitution of carboxymethyl-, hydroxyethyl-, sulfate-, or glucosyl-groups, oxidation of side chains, and limited degradation of the main chain. Despite the accumulation of the above evidence, the knowledge about the biochemical events of the early stage leading from the initial recognition of glucan molecules by the immune system of the host have been far from establishing a concept. This would be due to the difficulty of evaluation of biological activity of glucans *in vitro*. Incubation of glucan with macrophages *in vitro* generate little metabolic change. Some researchers recently suggested the existence of the (1→3)- β -D-glucan receptor on macrophages.^{9,10)} There is controversial evidence that bi-

ological activity of (1→3)- β -D-glucan was augmented by the incorporation into liposomes.¹¹⁾ Furthermore, there is little critical evidence to demonstrate the initial target cells of (1→3)- β -D-glucan and to clarify the kinetics of recognition of (1→3)- β -D-glucan by the cells.

Inactivation of (1→3)- β -D-glucan by an appropriate method at certain periods might give important information about the recognition mechanism of the glucan by the host. It is known that some chemical modification procedure significantly reduces the antitumor activity.¹²⁾ Because chemical modification has been considered to be impossible to apply *in vivo*, we conducted an enzyme digestion procedure by i.p. administration of (1→3)- β -D-glucanase at the period before completion of glucan recognition by the host. By observing the reduction of the antitumor activity, we hope to get kinetic data on glucan recognition *in vivo*. This paper shows the effect of (1→3)- β -D-glucanase treatment *in vivo* on antitumor activity of (1→3)- β -D-glucan.

Materials and Methods

Materials — Grifolan (GRN) obtained from liquid-cultured mycelium of *Grifola frondosa*

sa was prepared by the procedure described previously.¹³⁾ Carboxymethylated curdlan (CM-CUD) was prepared by reacting curdlan (1 g) with sodium monochloroacetate (1 g) in 0.5 N sodium hydroxide (100 ml) at 65 °C for 3 h. (1→3)- β -D-Glucanases were prepared from commercial enzyme preparations "Kitalase" (Keiai Kasei Co., Ltd.) and "Zymolyase 100 T" (Seikagaku Kogyo Co., Ltd.). Thioglycollate broth was obtained from Difco. (Detroit, Michigan, U.S.A.).

Preparation of (1→3)- β -D-Glucanase — Commercial kitalase preparation (50 g) was dissolved in 10 mM phosphate buffer (pH 5.0). After removal of the insoluble part by centrifugation (2500 r.p.m., 10 min), the resulting supernatant fluid was applied to a column of SP-Sephadex C-50 (3.0 $\times$ 25 cm) equilibrated with 10 mM phosphate buffer (pH 5.0). After washing with the same buffer, the column was eluted with a linear gradient of 10–100 mM phosphate buffer (pH 5.0). A (1→3)- β -D-glucanase-rich fraction was combined, concentrated by ultrafiltration (PM-10, Amicon), and equilibrated to 10 mM phosphate buffer (pH 6.8) by dialysis. The resulting enzyme preparation was applied to a column of hydroxyapatite, washed with the starting buffer, and eluted with a linear gradient of 0.01–0.1 M phosphate buffer (pH 6.8). The fractions containing (1→3)- β -D-glucanase were concentrated, and dialyzed against saline.

Enzyme Assay — GRN (0.25%) dissolved in PBS was incubated with the enzyme at 37 °C for 30 min. The reducing sugar produced was determined by literature methods.^{14,15)} 1 unit (U) of enzyme activity was defined as the amount of enzyme that produced 1 nmol of reducing sugar as glucose in 30 min under these conditions.

Opsonization of GRN — GRN (0.25%) dissolved in PBS was incubated with equal volumes of mouse serum in 30 min at 37 °C.

General Methods — Evaluation of antitumor activity and other methods were performed as described previously.¹⁶⁾

Results

Optimization of (1→3)- β -D-Glucanase Digestion

Fig. 1. Degradation of GRN by Various Amounts of KIT *in Vitro*

GRN (1 mg) was incubated with the various doses of KIT at 37 °C for 15 (—Δ—), 30 (—●—) and 60 (—○—) min in 1 ml of saline. Degree of the degradation was measured by the increase of reducing power.

Condition of GRN or CM-CUD *in Vivo* by Determining Enzymolytic Rate *in Vitro*

In order to apply (1→3)- β -D-glucanase to degrade (1→3)- β -D-glucan *in vivo*, several experiments were done for optimizing degradation conditions. We used two kinds of (1→3)- β -D-glucan: grifolan (GRN) and CM-curdan (CM-CUD), and two kinds of (1→3)- β -D-glucanase: kitalase and zymolyase. Zymolyase 100 T (ZYM) was used without further purification.

Fig. 2. Disappearance of the (1→3)- β -D-Glucanase Activity from the Peritoneal Cavity and Appearance in Serum after the Intraperitoneal Injection of KIT

KIT (8000 U) was injected intraperitoneally, and after appropriate time, the peritoneum was washed by 5 ml of saline. Simultaneously, blood was collected at appropriate time period after i.p. administration of KIT, and serum was prepared. The (1→3)- β -D-glucanase activity in peritoneal cavity (—○—) and serum (—●—) were measured by the increase of reducing power from GRN.

tion while kitalase (KIT) was partially purified to enrich (1→3)- β -D-glucanase activity as described in the Experimental section. The relative activity of the partially purified kitalase preparation (KIT) to degrade GRN : CUR : starch : islandican was 100 : 79 : 52 : 43. KIT did not hydrolyze dextran, methyl- α - and methyl- β -glucopyranosides. ZYM could degrade CM-CUD but not GRN. Hydrolysis of GRN by various amounts of KIT were shown in Fig. 1. More than 85% of GRN were hydrolyzed within 15, 30 or 60 min using 6000, 4000 or 2000 U of the enzyme, respectively. Increasing the reaction volumes decreased the rate of digestion; e.g., in 4 ml of total volume, hydrolysis was about 90, 50 or 30% in 8000, 4000 or 2000 U, respectively (data not shown). From these data, we decided to use 8000 U of KIT to degrade GRN *in vivo*.

After KIT solution was injected i.p., the rates of disappearance from the peritoneal cavity and appearance of the activity in blood were examined. More than 50% of the activity in the peritoneal cavity remained at 60 min, and 10% at 180 min (Fig. 2). On the other hand, the activity appeared in serum at 30 min after injection, and showed the highest activity at 120 min (Fig. 2).

There are possibilities that modification of GRN in the peritoneal cavity, such as oxidation by active oxygens released from the PMN and macrophage or binding of the proteins to GRN, affect the rate of digestion of GRN by KIT. To demonstrate this possibility, digestion of GRN

recovered from the peritoneal cavity and of GRN mixed with serum were performed. GRN was recovered from the peritoneal cavity at 24 h after injecting i.p. This GRN was degraded to 71% in comparison with native GRN. In addition, the opsonized GRN was degraded at the same rate as native GRN (data not shown). These results suggested that GRN would be degraded sufficiently by KIT *in vivo*. Precise experiments for CM-CUD and ZYM were not performed because of the structural similarity of these materials with GRN and KIT. These results suggested that the use of 8000 U of the enzyme solution would be sufficient to degrade GRN in the peritoneal cavity.

Effect of the (1→3)- β -D-Glucanase Treatment on the Antitumor Activity of GRN

Previous works have demonstrated that GRN shows the antitumor activity prior to as well as after transplantation of sarcoma 180. The optimum doses for single administration of GRN and CM-CUD, injected intraperitoneally at 7 d before transplantation of sarcoma 180 were 1000 and 750 μ g of GRN and CM-CUD, respectively. GRN (1 mg) was administered i.p. on -4 d, and various doses of (1→3)- β -D-glucanase were administered i.p. at 24 h after administration of GRN. As shown in Table I, the antitumor activity of GRN was abrogated by the treatment of the enzyme at doses of 8000 and 16000 U, but not significantly reduced at the doses under 2000 U. Following experiments

TABLE I. Effect of Various Doses of KIT Treatment on the Antitumor Activity of GRN^{a)}

Sample	KIT (unit)	Tumor weight ^{b)} (g, mean $\pm$ S.D.)	Inhibition ratio (%)	Complete regression ^{c)}
GRN	—	0.55 $\pm$ 0.99 ^{e)}	94 (94) ^{d)}	4/10
	16000	7.02 $\pm$ 7.54 ^{f)}	28 (11)	0/ 9
	8000	6.41 $\pm$ 6.05 ^{f)}	34 (19)	0/ 9
	4000	2.09 $\pm$ 3.52 ^{d)}	79 (74)	2/10
	2000	0.85 $\pm$ 1.40 ^{e)}	91 (89)	1/10
	1000	0.29 $\pm$ 0.62 ^{e)}	97 (96)	5/ 9
KIT	8000	7.91 $\pm$ 4.19 ^{f)}	19 (0)	0/10
Nil	—	9.74 $\pm$ 4.24	—	0/12

a) GRN (1 mg) was administered i.p. on 0 d, KIT (8000 U/400 μ l) was administered i.p. at the various doses at 24 h after the injection of GRN, and sarcoma 180 tumor cells (5×10^6) were inoculated s.c. on 4 d. b) The significance was evaluated according to Student's *t*-test. Significant difference from the corresponding control groups d) $p < 0.05$, e) $p < 0.001$, and from GRN f) $p < 0.001$. c) Number of cured mice/total mice. d) Inhibition ratio (%) calculated from KIT-administered groups.

TABLE II. Effect of the KIT Administration Time on the Antitumor Activity of GRN^{a)}

Sample	KIT (h)	Tumor weight ^{b)} (g, mean $\pm$ S.D.)	Inhibition ratio (%)	Complete regression ^{c)}
GRN	—	0.26 $\pm$ 0.46 ^{d)}	94	5/12
	0	5.41 $\pm$ 2.32 ^{e)}	7	0/ 9
	6	4.37 $\pm$ 2.76 ^{f)}	13	0/ 8
	12	2.82 $\pm$ 3.37 ^{g)}	44	1/ 8
	24	2.93 $\pm$ 2.70 ^{g)}	41	2/ 9
	48	0.98 $\pm$ 1.68 ^{d)}	80	2/ 9
	72	0.84 $\pm$ 2.10 ^{d)}	83	4/ 7
Nil	—	5.05 $\pm$ 1.66	—	0/11
GRN	—	0.26 $\pm$ 0.46 ^{d)}	94	5/12
Digest	—	9.78 $\pm$ 5.42 ^{e)}	0	0/10
KIT	—	7.91 $\pm$ 4.19 ^{e)}	18	0/10
Nil	—	9.74 $\pm$ 4.24	—	0/12

a) GRN (1 mg) was administered i.p. on 0 d, KIT (8000 U/400 μ l) was administered i.p. at the indicated hours after the injection of GRN, and sarcoma 180 tumor cells (5×10^6) were inoculated s.c. on 4 d. b) The significance was evaluated according to Student's *t*-test. Significant difference from the corresponding control groups d) $p < 0.001$, and from GRN e) $p < 0.001$, f) $p < 0.05$, g) $p < 0.01$. c) Number of cured mice/total mice.

have been done using 8000 U of KIT.

To examine the kinetics of KIT administration on the antitumor activity of GRN, KIT was administered at various hours (0, 6, 12, 24, 48 and 72 h) after administration of GRN (—4 d, 1 mg). Sarcoma 180 was inoculated on 0 d and antitumor activity was compared at 35 d after tumor transplantation. As shown in Table II, the antitumor activity of GRN was abrogated when the enzyme was administered at the same time as GRN. The antitumor activity of GRN appeared gradually, and at 48 and 72 h of administration, the activity was not distinguished from that of GRN itself. These results suggested that GRN injected into the peritoneal cavity was

degraded by KIT when the enzyme was administered in the early stage after administration of GRN. This result strongly suggests that recognition of GRN by certain host cells requires a relatively long period (a few days). Regeneration of antitumor activity in the cases of late KIT administered groups (48 or 72 h) resulted from either of the following reasons: a) the enzyme can not degrade GRN because of wide distribution of GRN in various organs or b) GRN had already been recognized by the host.

To confirm the above observation by another glucan-glucanase system, CM-CUD for (1 $\rightarrow$ 3)- β -D-glucan and ZYM for (1 $\rightarrow$ 3)- β -D-glucanase were used. As shown in Table III, the antitumor

TABLE III. Effect of ZYM Treatment on the Antitumor Activity of CM-CUD^{a)}

Sample	ZYM (h)	Tumor weight ^{b)} (g, mean $\pm$ S.D.)	Inhibition ratio (%)	Complete regression ^{c)}
CM-CUD	—	0.34 $\pm$ 0.58 ^{e)}	96	4/10
	0	8.80 $\pm$ 3.59 ^{f)}	4	0/10
	24	4.30 $\pm$ 4.47 ^{g)}	53	3/10
	48	0.04 $\pm$ 0.08 ^{e)}	99	6/10
	72	1.07 $\pm$ 3.11 ^{d)}	88	4/10
Nil	—	9.20 $\pm$ 6.74	—	0/17

a) CM-CUD (750 μ g) was administered i.p. at 7 d, ZYM (150 μ g/200 μ l) was administered i.p. at the indicated hours after the injection of CM-CUD, and Sarcoma 180 tumor cells (5×10^6) were inoculated s.c. on 0 d. b) The significance was evaluated according to Student's *t*-test. Significant difference from the corresponding control groups d) $p < 0.05$, e) $p < 0.001$, and from CM-CUD f) $p < 0.001$, g) $p < 0.01$. c) Number of cured mice/total mice.

TABLE IV. Effect of KIT or ZYM Treatment on Antitumor Activity of GRN and CM-CUD^{a)}

Glucan	Glucanase	Tumor weight ^{b)} (g, mean $\pm$ S.D.)	Inhibition ratio (%)	Complete regression ^{c)}
GRN	—	0.26 $\pm$ 0.46 ^{d)}	95	5/12
	KIT	5.41 $\pm$ 2.32 ^{e)}	—7	0/ 9
	ZYM	0.03 $\pm$ 0.06 ^{d)}	99	6/ 9
CM-CUD	—	0.18 $\pm$ 0.27 ^{d)}	96	5/10
	ZYM	4.14 $\pm$ 2.24 ^{e)}	18	0/ 9
	KIT	6.06 $\pm$ 3.49 ^{e)}	20	0/ 9
Nil	—	5.05 $\pm$ 1.66	—	0/11

a) GRN (1 mg) and CM-CUD (750 μ g) was administered i.p. on 0 d, KIT (8000 U/400 μ l) and ZYM (150 μ g/200 μ l) were administered i.p. immediately after the injection of GRN or CM-CUD, respectively, and Sarcoma 180 tumor cells (5×10^6) were inoculated s.c. on 4 d. b) The significance was evaluated according to Student's *t*-test. Significant difference from the corresponding control groups ^{d)} $p < 0.001$, and from GRN ^{e)} $p < 0.001$. c) Number of cured mice/total mice.

activity of CM-CUD was abrogated by the treatment of ZYM at 0 h, and regenerated at 48 h. These facts indicated a generalized phenomena that recognition of β -glucan by the host requires at least a few days.

To clarify whether the abrogation of the antitumor activities of GRN and CM-CUD were caused by degradation of these glucans by the (1 $\rightarrow$ 3)- β -D-glucanases in the peritoneal cavity, antitumor activity of GRN or CM-CUD were measured by treatment with these two (1 $\rightarrow$ 3)- β -D-glucanases. It is known that KIT digests both GRN and CM-CUD, but ZYM digests only CM-CUD. GRN or CM-CUD was administered i.p., and KIT or ZYM were administered i.p. immediately. Remaining antitumor activity was measured by growth inhibition of sarcoma

180. Sarcoma 180 was inoculated s.c. on 4 d after the administration of the glucan. As shown in Table IV, the antitumor activities of GRN were abrogated by the treatment of KIT but not of ZYM. On the other hand, antitumor activities of CM-CUD were abrogated by the treatments of both of these enzymes. These results confirm that only the treatment with (1 $\rightarrow$ 3)- β -D-glucanase which could act on (1 $\rightarrow$ 3)- β -D-glucans abrogates the antitumor activities of these glucans.

Effect of Administration-routes of GRN and/or KIT on Antitumor Activity of GRN

As described above, antitumor activity of GRN administered *via* i.p. route was abrogated by i.p. injection of KIT. In this section, we compare the effect of KIT-administered i.v. on the

TABLE V. Effect of i.v. Injection of KIT on the Antitumor Activity of GRN Administered by i.v.^{a)}

Sample	KIT (h)	Tumor weight ^{b)} (g, mean $\pm$ S.D.)	Inhibition ratio (%)	Complete regression ^{c)}
GRN	—	0.67 $\pm$ 1.32 ^{e)}	93 (92) ^{d)}	4/10
	0	4.37 $\pm$ 4.80 ^{d)}	55 (47)	0/ 9
	24	5.70 $\pm$ 6.73	42 (31)	1/ 8
	48	0.67 $\pm$ 0.75 ^{e)}	93 (92)	1/ 8
	72	1.53 $\pm$ 1.50 ^{e)}	84 (81)	1/ 6
KIT	—	8.22 $\pm$ 7.18 ^{f)}	16 (0)	0/ 8
Nil	—	9.74 $\pm$ 4.24	—	0/12

a) GRN (400 μ g) was administered i.v. on 0 d, KIT (8000 U/400 μ l) was administered i.v. at the indicated hours after the injection of GRN, and Sarcoma 180 tumor cells (5×10^6) were inoculated s.c. on 4 d. b) The significance was evaluated according to Student's *t*-test. Significant difference from the corresponding control groups ^{d)} $p < 0.05$, ^{e)} $p < 0.001$, and from GRN ^{f)} $p < 0.001$. c) Number of cured mice/total mice. d) Inhibition ratio (%) calculated from KIT administration groups.

TABLE VI. Effect of TG Treatment on the KIT Induced Abrogation of Antitumor Activity of GRN^{a)}

Sample	KIT (h)	Tumor weight ^{b)} (g, mean $\pm$ S.D.)	Inhibition ratio (%)	Complete regression ^{c)}
GRN	—	0.55 $\pm$ 0.99 ^{d)}	95	4/10
TG+GRN	—	1.94 $\pm$ 3.43 ^{d)}	80 (100) ^{d)}	3/ 9
	0	7.56 $\pm$ 2.46 ^{g)}	22 (27)	0/ 9
	24	3.16 $\pm$ 3.63 ^{e)}	68 (84)	1/ 9
	48	2.93 $\pm$ 4.47 ^{e)}	70 (87)	2/ 8
	72	3.80 $\pm$ 4.89 ^{f)}	61 (76)	0/ 8
TG	—	9.69 $\pm$ 5.32 ^{g)}	0 (0)	0/10
Nil	—	9.74 $\pm$ 4.24	—	0/12

a) TG (30 mg) was administered i.p. on -4 d, GRN (1 mg) was administered i.p. on 0 d, KIT (8000 U/400 μ l) was administered i.p. at the indicated hours after the injection of GRN, and sarcoma 180 tumor cells (5×10^6) were inoculated s.c. on 4 d.

b) The significance was evaluated according to Student's *t*-test. Significant difference from the corresponding control groups ^{d)} $p < 0.001$, ^{e)} $p < 0.05$, ^{f)} $p < 0.01$, and from GRN ^{g)} $p < 0.001$. ^{c)} Number of cured mice/total mice.

^{d)} Inhibition ratio (%) calculated from TG administration groups.

antitumor activity of GRN. GRN (1 mg) was injected intravenously, and KIT was injected i.v. at 0, 24, 48 or 72 h after administration of GRN, respectively. Sarcoma 180 was inoculated 4 d after injection of GRN and the tumor growth was compared. As shown in Table V, antitumor activities of the groups, which were KIT-administered at 0 and 24 h, were significantly reduced, but it is not as clear as the groups administered GRN and KIT i.p. These results suggested that the period required by the host to show antitumor activity is faster in the case of i.v. administration.

Effect of Thioglycollate Broth Treatment on the Kinetics of KIT Induced Abrogation of the Antitumor Activities of GRN

Previously, we have reported that i.p. injection of GRN elicited a significant numbers of peritoneal cells within a day. This phenomenon would be classified as an acute phase inflammatory response. Some of the irritant showed antitumor activity but the others did not. GRN probably acted first as the inducer of PEC (peritoneal exudated cells) and then as the activator of PEC. It is interesting to know whether the first signal (inducer) is replaced by another irritant.

It is known that injection of thioglycollate broth (TG) induces peritoneal exudated cells (PEC), but TG itself does not show antitumor activity. We examined the effect of TG injection on the KIT induced abrogation of the antitumor

activity of GRN. TG (30 mg) was administered i.p. on -4 d before administration of GRN. GRN (1 mg) was administered i.p., and KIT was injected i.p. at 0, 24, 48, and 72 h after administration of GRN, respectively. Sarcoma 180 was inoculated on 4 d after administration of GRN. As shown in Table VI, antitumor activity was only abrogated at 0 h, and regenerated already at 24 h. These results strongly suggested that some part of the activation mechanism of GRN could be replaceable by TG.

DISCUSSION

We wish to identify the initial events leading to the antitumor activity of (1 $\rightarrow$ 3)- β -D-glucans. The approach we used is based on limiting the effective time period of these glucans *in vivo* to induce antitumor activity by treatment with (1 $\rightarrow$ 3)- β -D-glucanase, kitalase (KIT) or zymolyase (ZYM). These glucanases were truly active *in vivo* because the antitumor activity of GRN was significantly reduced only by KIT treatment (antitumor activity of CM-CUD was reduced by KIT and ZYM) (Table IV). Furthermore, oxidative metabolism and/or binding of proteins to glucans did not significantly interfere with the action of glucanases. From these facts, the essential time period for these glucans to induce antitumor activity was demonstrated to be about two days in the case of intraperitoneal injection and this period was reduced to about

one day by the pretreatment with thioglycollate broth. These facts suggested that interactions of glucan with peritoneal exudated cells are important for the i.p. injected glucans to show antitumor activity. Intravenous administration of *P. acnes* or BCG induced penetration of macrophages to liver in which resident macrophages (kupffer cell) already existed. In the case of i.v. administration of GRN, similar kinetics of abrogation of antitumor activity by KIT were observed (Table V). These facts suggested that GRN also penetrates macrophages to liver and interaction of these macrophages would be important for antitumor activity.

Recently, phagocytic receptors on the cell surface of monocytes and/or macrophages against zymosan particles have been investigated.¹⁷⁾ These cell surface proteins might be one of the key factors for the initial events leading to antitumor activity. However, phagocytosis as well as oxidative burst of monocyte/macrophage by zymosan *in vitro* are known to proceed within minutes. Similar results were observed in the activation of PMN by CUD. The time period required by these reactions is shorter than that requiring induction of cells participating in antitumor activity as shown in this paper. Several factors underlying this phenomena are; kinetics of PEC exudation, velocity and capacity of PEC to recognize and respond to glucans, and existence of subpopulations responsible for interaction with glucans. These facts indicate the complexity of the activation mechanism leading to antitumor activity by (1→3)- β -D-glucans.

Antitumor activity of (1→3)- β -D-glucans are known to be mediated by the activation of host immune systems, and many kinds of cells are finally differentiated into cytotoxic or cytostatic stages. A part of maturation pathways are now understood to involve monokines and cytokines, such as Interleukines and Interferons. BRM (biological response modifier) are known to be the multiple cytokine inducers and some of the recombinant mediators are already applied to experimental animals and clinically. However, the results are not same as with the administration of just BRM. Glucan also stimulates macrophages to produce IL-1. However recombinant IL-1 also would not fully explain all of the

mechanism mediated by glucan administration. The data shown in this paper suggest that multiple signals from the glucan would be required to achieve fully activated stages of immune systems producing antitumor activity.

Acknowledgments The authors are grateful to Mr. N. Takata, Mr. K. Sakuraba, and Miss K. Katagiri for their technical assistance.

References

- 1) T. Taguchi, Y. Kaneko, and G. Chihara: Lentinan, *Biotherapy*, **2**, 509—521 (1988).
- 2) S. Fujimoto, K. Orita, T. Taguchi, K. Yoshida, T. Kimura, N. Ogawa, and H. Furue: Modern trends in immunostimulators, sizofiran, *Biotherapy*, **2**, 500—508 (1988).
- 3) J. Hamuro, M. Rollinghoff, and H. Wagner: Induction of cytotoxic peritoneal exudate cells by T-cell immune adjuvants of the β (1→3) glucan-type lentinan and its analogues, *Immunology*, **39**, 551—559 (1980).
- 4) J. Hamuro, M. Rollinghoff, and H. Wagner: β (1→3)-glucan-mediated augmentation of alloreactive murine cytotoxic T-lymphocytes *in vivo*, *Cancer Res.*, **38**, 3080—3085 (1978).
- 5) J. Hamuro, H. Wagner, and M. Rollinghoff: β (1→3)-glucans as a probe for T cell specific immune adjuvants, *Cell. Immunol.*, **38**, 328—335 (1978).
- 6) I. Suzuki, K. Hashimoto, and T. Yadomae: The effects of a highly branched β -1,3-glucan, SSG, obtained from *Sclerotinia sclerotiorum* IFO 9395 on the growth of syngeneic tumors in mice, *J. Pharmacobio-Dyn.*, **11**, 527—532 (1988).
- 7) E. R. Sherwood, D. L. Williams, R. B. McNamee, E. L. Joones, I. W. Browder, and N. R. DiLuzio: Enhancement of interleukine 1 and interleukine 2 production by soluble glucan, *Int. J. Immunopharmac.*, **9**, 261—267 (1987).
- 8) J. P. Fruehauf, G. D. Bonnard, and R. B. Herberman: The effect of lentinan on production of interleukin 1 by human monocytes, *Immunopharmacology*, **5**, 65—74 (1982).
- 9) M. J. Janusz, K. F. Austen, and J. K. Czop: Isolation of soluble yeast β -glucans that inhibit human monocyte phagocytosis mediated by β -glucan receptor, *J. Immunol.*, **137**, 3270—3276 (1986).
- 10) R. Goldman: Characteristics of the β -glucan receptor of murine macrophages, *Exp. Cell Res.*, **174**, 481—490 (1988).
- 11) T. Kawagoshi, I. Yamashita, K. Tazawa, K. Masuyama, M. Fujimaki, H. Ochiai, M. Maeda, and T. Honda: Inhibitory effect of lentinan entrapped in liposomes on hepatic metastasis in rats, *Biotherapy*, **2**, 1125—1130 (1988).
- 12) Y. Adachi, N. Ohno, M. Ohsawa, K. Sato, S. Oikawa,

- and T. Yadomae: Physicochemical properties and antitumor activities of chemically induced derivatives of antitumor glucan "Grifolan LE" from *Grifola frondosa*, *Chem. Pharm. Bull.*, **37**, 1838—1843 (1989).
- 13) N. Ohno, Y. Adachi, I. Suzuki, K. Sato, S. Oikawa, and T. Yadomae: Characterization of the antitumor glucan obtained from liquid-cultured *Grifola frondosa*, *Chem. Pharm. Bull.*, **34**, 1709—1715 (1986).
- 14) M. Somogyi: Notes on sugar determination, *J. Biol. Chem.*, **195**, 19—23 (1952).
- 15) N. Nelson: A photometric adaptation of the Somogyi method for the determination of glucose, *J. Biol. Chem.*, **153**, 375—380 (1944).
- 16) T. Takeyama, I. Suzuki, N. Ohno, S. Oikawa, K. Sato, M. Ohsawa, and T. Yadomae: Host-mediated antitumor effect of grifolan NMF-5N, a polysaccharide obtained from *Grifola frondosa*, *J. Pharmacobio-Dyn.*, **10**, 644—651 (1987).
- 17) J. P. Czop and K. F. Austen: A β -glucan-inhibitable receptor on human monocytes: its identity with the phagocytic receptor for particulate activators of the alternative complement pathway, *J. Immunol.*, **134**, 2588—2593 (1985).