COMPARATIVE STUDY OF ANTITUMOR ACTIVITY AND IMMUNOMODULATORY EFFECT OF TETRAHYDRO-2-FURANYL AND TETRAHYDRO-2-PYRANYL (1→3)- β -D-GLUCANS

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The effects of tetrahydro-2-furanyl- and tetrahydro-2-pyranyl-ethers of (1→3)- β -D-glucans from *Alcaligenes faecalis* var. *myxogenes* (IFO 13140) on the activities of macrophages and natural killer (NK) cells in either ICR or BALB/c mice were studied. The derivatives with strong antitumor activity against Sarcoma 180 in ICR mice induced higher macrophage tumoricidal activity (> 40%) than those with low antitumor activity (< 20%). All derivatives with high or low antitumor activity augmented NK cell activity. On the other hand, the derivatives which show the high antitumor activity against Meth-A in BALB/c mice enhanced macrophage tumoricidal activity, while those with low or no antitumor activity did not. NK cell activity was activated by the derivatives with or without the antitumor activity. Furthermore, certain derivative which has high antitumor activity against Sarcoma 180 in ICR mice did not increase serum component (LB) in ICR mice. These results indicate that the antitumor activity of the derivatives correlates only with the capacity to cause macrophage activation *in vivo*.

Keywords—tetrahydro-2-furanyl (1→3)- β -D-glucan; tetrahydro-2-pyranyl (1→3)- β -D-glucan; antitumor activity; macrophage tumoricidal activity; natural killer cell activity; serum component; ICR mouse; BALB/c mouse; L5178Y cell; YAC-1 cell

INTRODUCTION

Numerous reports deal with the antitumor activity of the polysaccharides, usually with a β -1,3-linkage.¹⁾ With few exceptions, however, the antitumor effect of these polysaccharides is not obtained in a syngeneic tumor system but is obtained almost in an allogeneic tumor system.²⁾ Previously we have reported that tetrahydro-2-furanyl- or tetrahydro-2-pyranyl- derivatives of (1→3)- β -D-glucans from *Alcaligenes faecalis* var. *myxogenes* (IFO 13140)³⁾ which shows the antitumor activity only against allogeneic tumor systems exert their inhibitory effects on a syngeneic tumor system (Meth-A in BALB/c mice).⁴⁾ These derivatives have not been found to exert any direct action on tumor cells.

Glucan has been shown to have antitumor properties, apparently as a result of its action on host-defense mechanisms to cancer.⁵⁾ The

macrophage is considered to be an important effector cell in host's defense against tumor growth. We reported that macrophages play a dominant role in the activity of (1→3)- β -D-glucan.^{6,7)} In addition to macrophages, NK cells are known to play a significant role in host's defense against tumor growth.⁸⁾ Since little is known about the antitumor action of the glucan derivatives, experiments were designed to study the effects of tetrahydro-2-furanyl- and tetrahydro-2-pyranyl-glucans on the activities of macrophages and NK cells, and also the increase of serum components when these glucans were given. The results of these experiments are summarized in this report.

MATERIALS AND METHODS

Glucans—(1→3)- β -D-Glucan (number-average degrees of polymerization, 540) was obtained

from the culture filtrate of *Alcaligenes faecalis* var. *myxogenes* (IFO 13140). Its lower-molecular-weight glucans and a variety of tetrahydro-2-furanyl- and pyranyl-ethers of the glucans were prepared by our method reported previously.^{3,4)} In this experiment, three pyranyl-derivatives (PG-1, 2, and 3) and three furanyl-derivatives (FG-1, 2, and 3) were used. Their physical property and antitumor activity have been reported as follows⁴⁾: The number-average degrees of polymerizations of PG-1, PG-2, PG-3, FG-1, FG-2, and FG-3 are 125, 125, 85, 97, 49, and 14, respectively. The average-number of tetrahydro-2-pyranyl or tetrahydro-2-furanyl groups substituted per anhydroglucose units of PG-1, PG-2, PG-3, FG-1, FG-2, and FG-3 are 0.08, 0.05, 0.06, 1.5, 1.7, and 1.1, respectively. PG-1 and FG-1 have low tumor inhibition ratios (49.2% and 30.4%, respectively) on Sarcoma 180 solid tumor in ICR mice when tested at the dose of 10 mg/kg for 10 d. PG-2, PG-3, FG-2, and FG-3 show high tumor inhibition ratios (100%, 100%, 84.4%, and 86.9%, respectively) with complete disappearance of the tumor in many animals under the same schedule as above; the inhibition ratios of PG-3 and FG-3 on Meth-A in BALB/c mice, when tested at the doses of 10 and 30 mg/kg for 10 d, respectively, are 86.6% and 83.3%, respectively. Other derivatives show no antitumor effect on this system. These glucans were supplied by Takeda Chemical Industries, Ltd., Osaka, Japan.

Mice—Female ICR mice, 6 to 8 week-old, were obtained from CLEA Japan Inc. Female BALB/c mice, 6 to 8 week-old and 15 week-old, were purchased from Charles River Co. Inc., Japan.

Target Tumor Cells—L5178Y lymphoma cells were induced by methylcholanthrene in adult DBA/2 mice and YAC-1 cells were from a Molony murine virus-induced lymphoma in a A/Sn mouse. Both cell lines were maintained in RPMI-1640 medium with 10% heat-inactivated fetal bovine serum (Grand Island Biological Co., Grand Island, N.Y.) and 50 μ g/ml of kanamycin (RPMI-FBS).

Labeling of Target Cells—Cultured L5178Y cells or YAC-1 cells were washed twice with RPMI-FBS and resuspended in RPMI-FBS at the concentration of 5×10^5 cells/ml. Both cells were labeled with ^3H -uridine (25 $\mu\text{Ci/ml}$) and incubated for 2 h at 37°C in 5% CO_2 in moist air. The cells were then washed three times with RPMI-FBS and resuspended at the required concentration in RPMI-FBS.

Effector Cells—Peritoneal-exudate cells (PEC) were harvested by washing with 5 ml of phosphate-buffered saline (pH 7.4, PBS) containing sodium heparin (2 units/ml). PEC were washed twice with PBS and suspended at the required concentration in RPMI-FBS. For preparation of macrophage monolayer, the suspension of PEC was seeded into plastic dishes (tissue culture dish: Costar, Cambridge, Mass.). After 1 h-incubation at 37°C in 5% CO_2 in moist air, nonadherent cells (NAC) were removed by repeated, intensive washing with RPMI-FBS. Adherent cells (AC) were recovered by adding RPMI-FBS and by gently scraping with a rubber policeman. More than 90% of the cells remaining in the dish had morphological characteristics of macrophages when stained with Gimsa solution. Each of AC and NAC was resuspended in RPMI-FBS after washing and was adjusted to the desired concentration.

Assay of Macrophage Activity—The activity of macrophages was tested in two ways.

(1) **Cytostatic Activity**: L5178Y cells were used as target cells *in vitro* at a ratio of macrophages to target cells of 5:1. AC (5×10^5 cells/ml) were incubated with L5178Y cells (1×10^5 cells/ml) at 37°C in 5% CO_2 in moist air. After 48 h, the effect of the macrophages on target cell proliferation was estimated with a phase-contrast microscope. The percentage macrophage cytostatic activity was calculated by the following formula: Macrophage cytostatic activity (%) = $[(A - B)/A] \times 100$, where A is the number of viable L5178Y cells per ml of culture after incubation with control macrophages and B is the number after incubation with treated macrophages.

(2) **Cytolytic Activity**: Cytolytic activity of

macrophages was measured by using ^3H -uridine and an emulsion scintillator.⁹⁾ Briefly, a mixture of 0.1 ml of ^3H -uridine labeled L5178Y cells (2×10^4 cells/ml) and 0.1 ml of AC (1×10^6 cells/ml) in a round bottomed plate was incubated for 18 h at 37°C in 5% CO_2 in moist air. After the plate was centrifuged at 1000 rpm for 10 min, 0.1 ml of supernatant was added to 0.9 ml of the emulsion scintillator and the mixture was counted for beta activity using a liquid scintillation counter. The percentage of cytolytic activity was calculated from the following formula:

$$\% \text{ of cytolytic activity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Spontaneous release was determined by incubation of 2×10^3 tumor cells in 0.2 ml of RPMI-FBS. Maximum release of radioactivity was determined by freezing and thawing of 2×10^3 tumor cells, four times in a dry ice-acetone bath. The amount of ^3H released spontaneously during incubation of target cells alone was below 40% of the maximum ^3H released.

Assay of NK Cell Activity—Activity of NK cells was measured by the following method. A mixture of 1×10^4 cells of ^3H -labeled YAC-1 cells and 5×10^5 cells of NAC per 0.2 ml in a round bottomed plate was incubated for 4 h at 37°C in 5% CO_2 in moist air. After centrifugation at 1000 rpm for 10 min, the amount of ^3H in supernatant was measured by an emulsion scintillator. The percentage of NK cell activity was calculated by the same formula as described in (2). Spontaneous release was determined by incubation of 1×10^4 tumor cells in 0.2 ml of RPMI-FBS. Maximum release of radioactivity was determined by freezing and thawing of 1×10^4 tumor cells, four times in dry ice-acetone bath. The amount of ^3H released spontaneously ranged from 15 to 25% of maximum ^3H released.

Assay of Serum Components—ICR mice were given a single *i.p.* injection of 100 mg/kg of glucans, and then at different time intervals mice were bled from the heart. The blood was allowed

to clot at 37°C for 30 min, and then sera were obtained by centrifugation at 3000 rpm for 10 min. The serum component, LB, was assayed by polyacrylamide gel electrophoresis.¹⁰⁾ Polyacrylamide gel electrophoresis on a gradient gel PAA 4/30 (Pharmacia Fine Chemicals, Uppsala, Sweden) was performed at 125 V for 15 h, using 90 mM Tris-borate buffer (pH 8.4) containing 2.5 mM ethylenediaminetetraacetic acid disodium salt (EDTA). The gel was stained for 30 min in 1% Amide Black in 7% acetic acid and then destained in 7% acetic acid.

Statistical Analysis—Student's *t* test was used for statistical analysis. All *p* values below 0.05 were considered to be statistically significant.

RESULTS

The Effects of the Glucans on Macrophage Activity in ICR Mice

First the kinetics of macrophage tumoricidal activity induced by the glucans was examined. PEC were harvested at various times after *i.p.* administration of the glucans and tested *in vitro* for their ability to evoke cytostatic activity of macrophages to inhibit L5178Y lymphoma cell proliferation. The cytostatic activity of the stimulated macrophages was measured on d 4, 7, 10, 14 and 18 after *i.p.* injection of 100 mg/kg the glucan. The results in Table I indicate that the cytostatic activity (> 40%) of macrophages from the mice appeared sooner after treatment with (1→3)- β -D-glucans than after treatment with PG-2, PG-3, FG-2, FG-3 with high antitumor activity against Sarcoma 180 being maximal on d 4 and 9, respectively. In contrast, PG-1 and FG-1 with low antitumor activity induced mild macrophage tumoricidal activity (< 20%) through 18 d after their treatment. In this experiment, macrophages from control untreated mice did not show any significant cytotoxicity at any time. These glucans that altered macrophage functions *in vivo* did not affect cell viability of tumor cells in the *in vitro* system. Moreover, incubation for up to 24 h with various concentrations of the glucans alone did not cause macrophage activation *in vitro*.

The results suggest that there is a strict relationship between the degree of macrophage-cytostatic activity induced by the glucans and the potency of their antitumor activity against Sarcoma 180 in ICR mice.

The Effects of the Glucans on NK Cell Activity in ICR Mice

On d 4, 7 and 10, NAC were prepared from ICR mice given *i.p.* the glucans at a dose of 100 mg/kg, and tested against YAC-1 cells in a 4 h ^3H -uridine release assay. As shown in Table I, all the glucans with low or high antitumor activity against Sarcoma 180 were found to be effective in enhancing the cytolytic activity of NAC. Since

TABLE I. Macrophage and NK Cell Activity of Peritoneal Cells from ICR Mice after Treatment *In Vivo* with the Glucans at Different Times

Glucan	Day 1	Day 4		Day 7		Day 10		Day 14	Day 18
	Macro-phage ^{a)} (%)	NK ^{b)} (%)	Macro-phage (%)	NK (%)	Macro-phage (%)	NK (%)	Macro-phage (%)	Macro-phage (%)	Macro-phage (%)
PG-1	6	23 ^{c)}	13	14	— 2	9	4 ^{d)}	7	9
PG-2	ND ^{e)}	6	9	13	29	26 ^{c)}	44 ^{d,f)}	31	ND
PG-3	ND	14	— 2	18	1	30 ^{c)}	50 ^{d,f)}	38	ND
FG-1	6	11	14	20 ^{c)}	16	14	19 ^{g)}	15	5
FG-2	ND	11	15	22 ^{c)}	21	14	42 ^{f,g)}	ND	ND
FG-3	ND	4	9	22 ^{c)}	32	14	45 ^{f,g)}	ND	ND

a) Macrophages were harvested on the indicated day after *i.p.* administration of the glucans at a single dose of 100 mg/kg and tested for their ability to inhibit L5178Y cells replication *in vitro* at a ratio of effector to target cells of 5:1.

b) NK cells were harvested on the indicated day after *i.p.* administration of the glucans at a single dose of 100 mg/kg and tested for their cytolytic activity to YAC-1 cells *in vitro* at a ratio of effector to target cells of 50:1.

c) $p < 0.05$, d) $p < 0.01$, value of PG-1 versus that of PG-2 or PG-3. e) ND, not determined. f) $p < 0.01$. g) $p < 0.01$, value of FG-1 versus that of FG-2 or FG-3.

FIG. 1. Polyacrylamide Gradient Gel Pattern of Serum Samples from ICR Mice Treated with the Glucans
A) Kinetics of the appearance of LB in serum of mice administered FG-3, (B) the pattern of sera from the mice on d 4 after administration of FG-1, FG-2, FG-3, PG-1, PG-2, and PG-3.

the cytolytic activity of NAC from the treated ICR mice was decreased upon *in vitro* incubation at 37°C and nylon wool column treatment did not reduce this activity and also YAC-1 cells were more sensitive than EL-4 cells to this activity, this cytolytic activity of NAC was due to that of NK cells.¹¹⁾ In this experiment, NAC from control untreated mice did not show any significant cytotoxicity at any time. These glucans that altered NAC functions *in vivo* did not affect cell viability of YAC-1 cells in the *in vitro* system. Also, incubation for up to 24 h with various concentrations of the glucans alone did not cause NAC activation *in vitro*. These results suggest that there is no correlation between the antitumor activity of the glucans against Sarcoma 180 and their capacity to enhance NK cell activity in ICR mice.

The Effects of the Glucans on the Increase of Serum Components in ICR Mice

FIG. 2. Kinetics of Macrophage and NK Cell Activations in Peritoneal Cavity of BALB/c Mice after *in Vivo* Treatment with PG-3

Macrophages were harvested on the indicated day after *i.p.* injections of PG-3 to BALB/c mice, 6 to 8 week-old, and tested for their cytolytic activity to L5178Y cells *in vitro* at a ratio of effector to target cells of 50:1 (○). NK cells were harvested on the indicated day after *i.p.* injection of PG-3 to BALB/c mice, 15 week-old, and tested for their cytolytic activity to YAC-1 cells at a ratio of effector to target cells of 50:1 (●). The significance of this observation was $p < 0.01$.

Sera were prepared from ICR mice at different time intervals after *i.p.* injection of the glucans at a dose of 100 mg/kg and then subjected to polyacrylamide gel electrophoresis on a PAA 4/30 gradient gel. Figure 1A shows kinetics of the appearance of LB in serum of mice administered FG-3. The amount of LB reached peak on d 4 after its injection and then gradually decreased, returning to similar level to that in control mice on d 10. The patterns of sera from FG-1, FG-2, PG-1, and PG-2 treated mice on gel electrophoresis were close to that obtained from FG-3 treated mice. LB of three protein components mildly increased in sera of ICR mice administered PG-1, PG-2, FG-1, FG-2, and FG-3 on d 4, but not PG-3 (Fig. 1B). PG-3 did not increase three protein components through 18 d after its injection. These results indicate that the increase of three serum proteins cannot be the indicator of antitumor activity, at least, in the case of these glucan derivatives.

The Effects of the Glucans on the Activities of Macrophages and NK Cells in BALB/c Mice

To explore the effect of the glucans on macrophage activity in BALB/c mice (6–8 week-old), AC were tested against L5178Y cells in a 18 h ³H-uridine release assay. The kinetics of macrophage cytolytic activity induced by PG-3 was examined. PEC were harvested at various times after administration of PG-3 and tested *in vitro* for its ability to evoke cytolytic activity of macrophages to inhibit L5178Y cells. The cytolytic activity of the stimulated macrophages was measured on d 1, 4, 7, 10 and 14 after *i.p.* administration of 50 mg/kg PG-3 on 2 consecutive day. The result in Fig. 2 shows that PG-3 activated macrophage cytolytic activity at 10 and 14 d after the last injection. Next, macrophage cytolytic activity of the glucans was also examined at 10 d after *i.p.* injection of PG-2, PG-3 and FG-3 at doses of 50, 50, and 150 mg/kg on 2 consecutive day, respectively. As shown in Table II, PG-3 and FG-3 with antitumor activity against Meth-A in BALB/c mice augmented macrophage cytolytic activity, while (1→3)- β -D-glucan and PG-2 with no antitumor activity

against Meth-A in BALB/c mice did not. These results indicate that the formation of the tetrahydro-2-furanyl or tetrahydro-2-pyranyl derivatives of (1→3)- β -D-glucans is capable of eliciting greater stimulation of macrophage activation and possesses more potent antitumor activity against Meth-A in BALB/c mice than the parent glucans.

The effect of PG-2 and PG-3 on NK cell activity in BALB/c mice, 15 week-old, was also examined. NAC were prepared from BALB/c mice given PG-2 or PG-3 at a dose of 50 mg/kg on 2 consecutive day and tested against YAC-1 cells in a 4 h ^3H -uridine release assay. The results in Fig. 2 indicate that antitumor active PG-3 augmented the cytolytic activity of NAC at 7 d after the last injection. The peak of NK cell activity for PG-3 occurred 3 d prior to its peak of macrophage activation. The pattern of cytolytic activity of NAC from antitumor nonactive PG-2 treated mice was close to that obtained from PG-3 treated mice. Table II shows the results of cytolytic activity of NAC from PG-2 and PG-3 treated mice on 7 d after their *i.p.* injections. The cytolytic activity of NAC from treated BALB/c mice was due to that of NK cells, because this activity was decreased by *in vitro* incubation at 37°C and was not decreased by nylon wool col-

umn treatment and furthermore YAC-1 cells were more sensitive than EL-4 cells to this activity.¹¹⁾ In this experiment, macrophages or NK cells from control untreated BALB/c mice did not show any significant cytotoxicity within 18 or 4 h incubation periods, respectively. Furthermore, incubation for up to 24 h with various concentrations of the glucans alone did not cause any macrophage and NK cell activation *in vitro*.

DISCUSSION

Previously we found that tetrahydro-2-furanyl- and tetrahydro-2-pyranyl-ethers of (1→3)- β -D-glucan from *Alcaligenes faecalis* var. *myxogenes* (IFO 13140) exert their antitumor effect on a syngeneic tumor system (Meth-A in BALB/c mice) as well as an allogeneic tumor system (Sarcoma 180 in ICR mice).⁴⁾ The antitumor activity of polysaccharides seems to be based on the activation of host's defence against tumors.⁵⁾ The differences of antitumor potency among (1→3)- β -D-glucan and its derivatives are probably due to their different activation of host's response. The present investigations concern with the antitumor action of the tetrahydrofuranyl- and tetrahydropyranyl-ethers of (1→3)- β -D-glucan, especially their effects on the activities of macrophages and NK cells, and the increase of

TABLE II. Macrophage and NK Cell Activity of Peritoneal Cells from BALB/c Mice after Treatment *in Vivo* with the Glucans

Glucan	Dose (mg/kg $\times$ 2 d)	% cytotoxicity	
		Macrophages ^{a)}	NK cells ^{b)}
None		3	5
PG-2	50	— 2	32 ^{c)}
PG-3	50	34	39
FG-3	150	32	ND ^{d)}
(1→3)- β -D-Glucan	50	7	ND

a) Macrophages were harvested on 10 d after the last *i.p.* injection of the glucans and tested for their ability to inhibit L5178Y cells replication *in vitro* at a ratio of effector to target cells of 50:1.

b) NK cells harvested on 7 d after the last *i.p.* injection of the glucans and tested for their cytolytic activity to YAC-1 cells *in vitro* at a ratio of effector to target cells of 50:1.

c) Values above 30% inhibition; $p < 0.01$.

d) ND, not determined.

serum component (LB) when they are given into two mouse strains. The effects of the glucans on the activities of macrophages and NK cells in ICR mice were examined. The results indicated that the antitumor efficacy of the glucans *in vivo* corresponded to the potency of macrophage activation. FG-2, FG-3, PG-2, and PG-3 with high antitumor activity against Sarcoma 180 induced macrophages with higher cytostatic activity (> 40%) than those (< 20%) induced by FG-1 and PG-1 with lower antitumor activity. The optimum response occurred at different time intervals after injection of test glucans. The peak of macrophage activity for (1→3)- β -D-glucan occurred at 4 d after injection,⁷⁾ for FG-2, FG-3, PG-2, and PG-3 at 10 d. At present, the reason for these different kinetics is unclear. On the other hand, NK cell activity did not correspond to the antitumor efficacy of the glucans *in vivo*. All the glucans even without antitumor activity *in vivo* enhanced NK cell activity.

Maeda *et al.*¹⁰⁾ reported that the concentration of three protein components, named LA, LB, and LC, markedly increase in mouse serum soon after administration of lentinan, an antitumor polysaccharide from *Lentinus edodes*. They also reported a close relation between increase in the level of these protein components and the antitumor activity of the polysaccharide. Accordingly, we studied the correlation between the antitumor activity of the glucan derivatives and serum components. Three protein components markedly increased in the serum of ICR mice administered original (1→3)- β -D-glucan,⁷⁾ but FG-1, FG-2, FG-3, PG-1, and PG-2 mildly increased only LB; its amount reached peak on d 4 after injection, and gradually decreased, returning to similar level to that in control mice on d 10. Of great surprise were the findings that three protein components did not increase in the serum of mice administered PG-3 with antitumor activity against Sarcoma 180 in ICR mice and also that LB increased in the serum of mice administered PG-1 and FG-1 without antitumor activity. These unexpected findings are perplexing and one interpretation for these findings may be that appearance of such

serum components is not always causally related with antitumor effect of polysaccharide, although it is possible that the antitumor action of these glucan derivatives differs from that of other antitumor polysaccharides. Further study of this is in progress.

The effects of the glucan derivatives on macrophage and NK cell activities in BALB/c mice were also studied. The antitumor activity of polysaccharides differs among mouse strains.¹²⁾ BALB/c mice are especially weak responders to antitumor polysaccharides. PG-3 and FG-3 with the antitumor activity on Meth-A in BALB/c mice augmented macrophage tumoricidal activity, while (1→3)- β -D-glucan and its derivatives with low antitumor activity did not. These results indicate that there is a close relationship between the antitumor activity of the glucans and stimulation of macrophage activity even in BALB/c mice.

No such correlation was observed in NK cell activity. The glucan derivatives with or without the antitumor activity against Meth-A in BALB/c mice enhanced NK cell activity in BALB/c mice.

From the data reported in this article it is obvious that macrophages are important effectors of furanyl- and pyranyl-glucans tumor inhibition on either allogeneic or syngeneic tumor systems. Although the mechanism of action of the glucan derivatives needs further study, this macrophage assay could serve as a reliable cell-mediate assay for evaluating potential polysaccharide derivatives.

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