Failure in Antitumor Activity by Overdose of an Immunomodulating β -Glucan Preparation, Sonifilan

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Schizophyllan (SPG, Sonifilan) is a soluble (1 $\rightarrow$ 3)- β -D-glucan, used as a biological response modifier (BRM) with radiation therapy for cancer treatment in Japan. The mechanism of SPG mediated antitumor activity is thought to be *via* immune stimulation, which includes cytokine production, hematopoietic response, and so on. In this paper, we found that the activity of SPG was quite long-lived and an overdose significantly failed to display the antitumor activity. To demonstrate the mechanism several parameters were examined using a high dose of SPG administration as follows: i) the effect on vascular permeability *in vivo*, ii) the priming effect on tumor necrosis factor (TNF- α) production *in vivo*, iii) the effect on macrophage adherence to plastic plate *in vitro*, and iv) anti-Sarcoma 180 antibody production *in vivo*. It was evident that vascular permeability and anti-Sarcoma 180 antibody production remained unchanged, but TNF- α production and adherence to a plastic plate was significantly reduced by a high dose of SPG. These facts strongly suggested that modulation of the cytokine syntheses and the leukocyte traffic would be the causative mechanisms of the failure of antitumor activity by an overdose of SPG.

Key words sonifilan (SPG); antitumor activity; overdose β -glucan; biological response modifier (BRM)

(1 $\rightarrow$ 3)- β -D-Glucan is a group of immunomodulators, some of which have been used clinically. We have long been working on analyzing the relationship between the structure and activities of (1 $\rightarrow$ 3)- β -D-glucan.^{1,2)} Schizophyllan (SPG, Sonifilan) is a soluble (1 $\rightarrow$ 3)- β -D-glucan, used as a biological response modifier (BRM) with radiation therapy for cancer treatment in Japan.^{3,4)} SPG was isolated and purified from the culture supernatant of a fungus, *Schizophyllum commune*. The mechanism of SPG mediated antitumor activity is believed to be *via* immune stimulation, which include cytokine production, hematopoietic response, modulation of cell surface antigen molecules essential for signal transduction, complement activation, antigen processing and presentation, and so on.^{5–8)} Beta-glucan receptor(s) might also contribute, at least in part, to the activity. The molecular mechanism of the activity, however, has not yet been fully clarified.

Establishment of standard cancer therapy, which includes chemotherapy, radiation, surgery, and biotherapy, is an issue of primary importance worldwide. Even though numerous studies, both basic and clinical, have been performed or planned for this purpose, there remains much to be done. Efficacy of the biotherapy, unfortunately, is still inadequate. It has recently been clinically established that cancer cells often express antigen, capable of inducing antitumor immunity. Optimization of the usage of BRMs to enhance the antitumor immunity would be beneficial.

Suda *et al.* examined the fate of (1 $\rightarrow$ 3)- β -D-glucan in mice using a soluble highly branched (1 $\rightarrow$ 3)- β -D-glucan, SSG, obtained from the culture filtrate of *Sclerotinia sclerotiorum* IFO 9395, and found that SSG deposited in organs, mainly liver and spleen, for very long period of time without causing major structural changes.^{9,10)} SSG collected from liver retained significant antitumor activity. We have found the similar fate of (1 $\rightarrow$ 3)- β -D-glucan in mice, in soluble and particulate forms. We have also shown that the antitumor activity gradually disappeared independent of the content of

(1 $\rightarrow$ 3)- β -D-glucan in mice, and suggested that several inactivation mechanisms may contribute to this.^{11,12)} The fate of (1 $\rightarrow$ 3)- β -D-glucan is quite long compared with other bioactive materials, thus the action mechanism might also include special and characteristic steps.

During the course of this project we found it interesting that a higher dose of SPG significantly failed to show the antitumor activity. This paper describes and discusses the antitumor effect of a higher dose of SPG and the proposed mechanism causing the failure.

MATERIALS AND METHODS

Materials SPG in triple helix conformation for clinical use was generously provided by Kaken Pharmaceutical Co., Tokyo. Zymosan (ZYM) and lipopolysaccharide (LPS) from *Escherichia coli* O111, extracted by the phenol–water procedure, was provided by Sigma Co.

Animals Specific-pathogen-free male ICR mice were purchased from Japan SLC (Shizuoka, Japan), housed under specific-pathogen-free conditions and used at 5–10 weeks of age.

Antitumor Activity Antitumor activity against the solid form of Sarcoma 180 tumor was measured by the method described previously.¹³⁾ Sarcoma 180 cells (5×10^6) were inoculated subcutaneously into the right groin of ICR mice (day 0). SPG was diluted with saline and administered intraperitoneally every other day (3 times). The timing of administration of SPG was altered in each experiment. After 5 weeks, the mice were killed and tumors were weighed.

Measurement of (1 $\rightarrow$ 3)- β -D-Glucan by Fungitec G Test MK The activation of factor G (limulus reactivity) by (1 $\rightarrow$ 3)- β -D-glucan was measured by a chromogenic method using a (1 $\rightarrow$ 3)- β -D-glucan specific reagent (Fungitec G-test MK, Seikagaku Corp., Tokyo), which eliminates factor C.^{14,15)} (1 $\rightarrow$ 3)- β -D-Glucan was dissolved in 0.3 N NaOH

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(1 mg/ml) and diluted with 0.01 N NaOH. Sample solution was used directly for the limulus reaction without neutralization. Diluted NaOH was confirmed to be usable for the limulus reaction because of the high buffer action of the reagent. Reactions were performed in flat-bottomed 96-well Toxipet plates 96F (Seikagaku Corp.) as follows. Samples (50 μ l) were placed in the wells, and the Fungitec G test MK reagent (50 μ l) was added to each well. The plate was incubated at 37 °C, and during incubation the absorbance at 405 nm (reference: 492 nm) was measured kinetically using a microplate reader (Wellreader SK603, Seikagaku Corp.). Disposable plastic materials for tissue culture or clinical use were employed, and all glassware was sterilized at 260 °C for 3 h. All operations were performed in triplicate under aseptic conditions.

Measurement of Vascular Permeability Several doses of SPG were administered to mice before 1, 3 or 7 d of vascular permeability assay.¹⁶⁾ The stimulant (*ca.* 100 μ g per mouse) ZYM was i.p. injected to mice, and thirty minutes thereafter, aqueous solution of Evans blue (Sigma E-2129; 10 mg/ml in saline) was injected i.v. (0.2 ml), unless otherwise stated. Thirty minutes later, mice were sacrificed and the peritoneal exudate fluids were collected by washing with 4 ml of phosphate buffered saline (PBS). After centrifugation to remove any debris, concentration of Evans blue in the peritoneal cavity was measured at 630 nm.

ELISA for Cytokine Determination Tumor necrosis factor- α (TNF- α) was detected using an indirect enzyme-linked immunosorbent assay (ELISA). A 96 well plate (Sumitomo Bakelite Co., Tokyo) was coated with rat anti-mouse TNF- α mAb (Pharmingen, CA) in 0.1 M bicarbonate buffer (pH 9.6) by incubating at 4 °C overnight. The plate was washed with PBS containing 0.05% Tween 20 (Wako Pure Chemical Co.) (PBST) and blocked with 0.5% bovine serum albumin (Sigma) (BPBST) at 37 °C for 40 min. After washing, the plate was incubated with rm-TNF- α (R & D Systems, MN) or 50 μ l of samples at 37 °C for 40 min. The plate was washed with PBST and blocked with BPBST at room temperature for 10 min, then treated with rabbit anti-mouse TNF- α polyclonal antibody (Genzyme, MA). It was next treated with peroxidase-labeled anti-rabbit IgG (Genzyme, MA) and a TMB substrate system (KPL Inc., MD). Color development was stopped with 1 N phosphoric acid and the optical density was measured at 450 nm.

Preparation of Peritoneal Exudate Cells Resident peritoneal exudate cells (PECs) were collected from the peritoneal cavity of mice by washing twice with 5 ml of Hank's balanced salt solution (HBSS; Nissui Seiyaku Co., Tokyo) containing heparin (5 U/ml). PECs were centrifuged to pellet the cells. Pellets were washed twice and resuspended in an ice-cold RPMI 1640 medium (Nissui) supplemented with *N*-hydroxyethylpiperazin-*N*-2-thansuifonate (HEPES, 5 mM), penicillin G (100 U/ml) and streptomycin (100 μ g/ml). The cells were cultured in 96-well flat-bottomed plates (Sumitomo Bakelite Co., Ltd.) at 1×10^5 per well in 100 μ l of culture medium. To ensure adherence, cells were cultured for at least 30 min, then washed twice with warmed RPMI 1640 medium. Quantity of adherent cells was determined by bicinchoninic acid (BCA) protein assay and represented as protein.

Measurement of Anti-Sarcoma 180 (Anti-S180) Anti-

Table 1. Antitumor Activity of Various Doses of SPG in ICR Mice

Day	Dose (μ g/mouse $\times$ 3)	Tumor weight (g, mean $\pm$ S.D.)	Inhibition (%)	CR/total
-90	50	2.2 $\pm$ 1.5	62.7	4/8
	250	0.7 $\pm$ 0.7	88.1	3/7
	1000	0.2 $\pm$ 0.4	96.6	5/9
	2000	0.7 $\pm$ 1.0	88.1	1/10
-60	50	2.0 $\pm$ 3.6	66.1	5/9
	250	0.4 $\pm$ 1.0	93.2	5/7
	1000	0.8 $\pm$ 1.1	86.4	1/7
	2000	0.9 $\pm$ 1.0	84.7	3/9
-30	50	2.2 $\pm$ 3.3	62.7	5/10
	250	0	100.0	10/10
	1000	1.5 $\pm$ 1.4	74.6	3/10
	2000	2.7 $\pm$ 3.7	54.2	1/10
+ 7	50	0.6 $\pm$ 1.5	89.8	8/10
	250	0.2 $\pm$ 0.5	96.6	9/10
	1000	1.3 $\pm$ 2.1	78.0	2/10
	2000	2.6 $\pm$ 1.9	55.9	1/10
Nil	0	5.9 $\pm$ 3.1	0.0	0/10

SPG was administered three times every other day -90, -60, -30 d before or 7 d after Sarcoma 180 inoculation. Doses of SPG were 50, 250, 1000, 2000 μ g/mouse ($n=7-10$). Sarcoma 180 tumor cells (5×10^6 cells/mouse) were inoculated subcutaneously into the right groin of ICR mice on day 0. Five weeks after tumor inoculation, the mice were sacrificed and tumors were weighed. CR, complete regression.

body Sarcoma 180 tumor cells (5×10^6 cells/mouse) were inoculated subcutaneously into the right groin of ICR mice (day 0). Two, 1 mg or 200 μ g of SPG was administered on days 7, 9, and 11 after the inoculation. Three weeks after the tumor inoculation, the mice were sacrificed and the sera were collected. Anti-S180 antibody levels in sera were measured by ELISA using Triton X-100 solubilized Sarcoma 180 coated plate (Nunc, Denmark) with anti-mouse IgG-horse-radish peroxidase (HRP).

Statistics Statistical evaluation in all experiments was performed by Student's *t*-test. A value of $p < 0.05$ was considered significant.

RESULTS

Dose Response of the Antitumor Activity of SPG on General Assay Protocol Sarcoma 180 was administered subcutaneously on day 0 and a dose of SPG from 50—2000 μ g/mouse/d was intraperitoneally administered on days 7, 9, and 11. This administration protocol is widely used as the most effective schedule for (1 $\rightarrow$ 3)- β -D-glucan. Tumor mass of each mouse was weighed on day 35 and the results are summarized in Table 1 (shown as +7 group). By the +7 administration schedule, the strongest activity was shown at the dose of 250 μ g/mouse/d $\times$ 3 and the number of tumor free mice was 9 in 10. The groups administered lower as well as higher doses of SPG showed lower activity, assessed by both mean tumor weight and the number of tumor free mice. It is of note that the mice administered 2000 μ g/mouse/d $\times$ 3 of SPG showed only 55.9% tumor growth inhibition. Antitumor activity of SPG is believed to be mediated by immune stimulation,⁵⁾ thus a certain step(s) of the pathway(s) might have been inhibited or have failed as a result of the overdose.

Kinetics of the Antitumor Activity of SPG In a previous study we showed that the activity of SPG was long-lived and was demonstrated in prophylactic as well as therapeutic

Chart 1. Protocol of Antitumor Activity for Prophylactic Administration of SPG

Table 2. SPG Concentration in Mouse Serum

Dose ($\mu\text{g}/\text{mouse} \times 3$)	1 d ($\mu\text{g}/\text{ml}$)	1 month ($\mu\text{g}/\text{ml}$)
2000	731.5	5.0
1000	420.6	4.0
50	9.9	N.D.

SPG was injected i.p. into ICR mice. After 1 d or 1 month, sera were collected, and SPG concentration in sera was measured by Limulus G-Test.

treatments.¹¹⁾ To examine the kinetics of the activity in more detail, various doses of SPG were administered 90, 60, or 30 d prior to the tumor transplantation and the activity was measured by the method shown above (see Chart 1). The results are summarized in Table 1. At the dose of $50 \mu\text{g}/\text{mouse}/\text{d} \times 3$, the activity by prophylactic treatment was weak and disappeared when administered at -30 d . The strongest activity was shown at the dose of $250 \mu\text{g}/\text{mouse}/\text{d} \times 3$ in all periods of the experiment. Of interest was that the group administered $2 \text{ mg}/\text{mouse}/\text{d} \times 3$ showed strong activity by prophylactic treatment, especially at -90 d . These facts demonstrated that the kinetics of the strength in antitumor activity was clearly opposite between higher and lower doses.

Concentration of SPG in Sera (1 $\rightarrow$ 3)- β -D-Glucan is known to deposit mainly in liver and spleen for a very long period of time. Serum concentration of SPG in various administration schedules was measured by (1 $\rightarrow$ 3)- β -D-glucan specific limulus G-test. As shown in Table 2, the concentration of SPG was $9.9 \mu\text{g}/\text{ml}$ in the mice on day 1 of $50 \mu\text{g}/\text{mouse}/\text{d} \times 3$ administration, but the level fell below the detection limit at 1 month. In contrast, the concentration was $731.5 \mu\text{g}/\text{ml}$ in the mice on day 1 of $2000 \mu\text{g}/\text{mouse}/\text{d} \times 3$ administration, and was reduced to $5.0 \mu\text{g}/\text{ml}$ at 1 month. Interestingly, the concentration of SPG in $2000 \mu\text{g}/\text{mouse}/\text{d} \times 3$ administered mice at 1 month was comparable to that in $50 \mu\text{g}/\text{mouse}/\text{d} \times 3$ administered mice on day 1. Considering the data shown in Table 1, blood concentration of around $10 \mu\text{g}/\text{ml}$ would be the optimum concentration for antitumor activity.

Immune and Inflammatory Parameters of SPG Administered Mice Failure of the antitumor activity of SPG by overdosing might be related to a certain parameter of immune and/or inflammatory response. We have been working on the relationship between structure and function of (1 $\rightarrow$ 3)- β -D-glucan. In contrast to the strong antitumor activity, SPG showed weaker or only slight activity compared with other (1 $\rightarrow$ 3)- β -D-glucans when assessed by: i) splenomegaly, ii)

Fig. 1. Effect of High Dose of SPG on Vascular Permeability

Twenty, 200, or $2000 \mu\text{g}$ of SPG was i.p. injected to mice. After A) 1, B) 3 or C) 7 d, ZYM was i.p. injected to the animals, and thirty minutes thereafter, an aqueous solution of Evans blue was injected i.v. After thirty minutes, mice were sacrificed and the peritoneal exudated fluid was collected by lavaging with PBS. After centrifugation to remove any debris, the concentration of Evans blue in the peritoneal cavity was measured at 630 nm. * $p < 0.001$ compared with control, ** $p < 0.01$ compared with control, *** $p < 0.001$ compared with ZYM.

exudation of leukocytes to the peritoneal cavity, iii) cytokine synthesis of macrophages, such as $\text{TNF-}\alpha$ and IL-6, even in the presence of $\text{IFN-}\gamma$, and iv) expression of the inducible nitric oxide synthetase on macrophages *in vivo* and *in vitro*; thus these parameters would not be usable to analyze the failure of antitumor activity appearing solely by an overdose of SPG.^{17,18)} We chose parameters such as vascular permeability, cytokine synthesis, adherence of peritoneal exudate cells, and anti-S180 antibody response to identify the underlying mechanism. Figure 1 shows vascular permeability of SPG administered mice induced by ZYM, but the activity was not or was only slightly modulated by prior SPG administration. Figure 2 shows the priming effect of $\text{TNF-}\alpha$ synthesis. Various concentrations of SPG were administered on day -7 and LPS was administered on day 0. The sera were collected 1 h later and $\text{TNF-}\alpha$ concentration in them was measured by ELISA. The priming effect was reduced significantly at doses of 250 or $1000 \mu\text{g}/\text{mouse}$. Figure 3 shows plastic adherence of peritoneal exudate macrophage, and the adherence was significantly inhibited by a high concentration of SPG. Fig-

Fig. 2. Dose Effect of SPG on Priming Effect to LPS Elicited TNF- α Synthesis

Twenty, 100, 250 or 1000 μ g of SPG was i.p. injected to mice. LPS (10 μ g) was i.v. injected 7 d later and the sera were collected 1 h thereafter. TNF- α levels in sera were detected by ELISA. * p <0.05 compared with SPG 100 μ g i.p.

Fig. 3. Effect of SPG on Plastic Adherence by Peritoneal Macrophage

Two or 10 mg/ml of SPG (100 μ l) was added to a 96 well tissue culture plate (Sumitomo Bakelite Co., Ltd., Tokyo). Peritoneal exudated cells (1×10^6 /ml suspended in RPMI 1640 (Sigma), 100 μ l) were added and incubated in the humidified CO₂ incubator at 37°C for 30 min. After the incubation, each well was washed with warmed RPMI 1640 and the number of adherent cells were calculated as the amount of protein by BCA (Pierce Co., Rockford, IL) method. * p <0.001.

Fig. 4. Effect of SPG on Anti-S180 Antibody Response in Sarcoma 180 Inoculated Mice

Sarcoma 180 tumor cells (5×10^6 cells/mouse) were inoculated subcutaneously into the right groin of ICR mice. Two, 1 mg or 200 μ g of SPG was administered on days 7, 9, and 11 thereafter. Three weeks after the tumor inoculation, the mice were sacrificed and sera were collected. Anti-S180 antibody levels in sera were measured by ELISA using Triton X-100 solubilized a Sarcoma 180 coated plate (Nunc, Denmark) with anti-mouse IgG-HRP. * p <0.01, ** p <0.05.

Figure 4 shows the effect of SPG on anti-S180 antibody production in mice; SPG did not alter the antibody titer in Sarcoma 180 bearing mice. These facts strongly suggested that several parameters such as cytokine synthesis and adherence were negatively modulated by an overdose of SPG.

DISCUSSION

We clearly demonstrated here that i) activity of SPG was quite long-lived when maintained in the optimum concentration in blood, and ii) an overdose of SPG inhibited the antitumor activity. Several mechanisms might be operating, but at least any error in leukocyte traffic to the tumor, relation to the adhesion molecule and to the cytokine production would be important.

Adhesion molecules have various physiological functions, such as extravasation of the inflammatory cells, co-stimulatory signals from the antigen presenting cell to T cells, receptors of microbes, and so on.^{19–21} One candidate molecule of the β -glucan receptor, CR3, is a member of the integrin family (CD11b/CD18),^{22–24} and is distributed on phagocytic cells and important for many steps in adhesion.^{25–28} It is well demonstrated that phagocytosis, H₂O₂ release, and cytokine release of the particulate (1 $\rightarrow$ 3)- β -D-glucan by macrophage was inhibited by SPG.²⁹ In this paper, we found that the adherence of macrophage to the plastic plate was inhibited by SPG. These facts strongly suggested that three phenomena, i) adherence, ii) recognition of particulate glucan, and iii) inhibition of functions of phagocytes by SPG were induced by closely related surface molecules of leukocytes. In addition, frequency of the infiltration of leukocytes to the tumor tissue, assessed by histochemical analysis, was significantly reduced by an overdose of SPG (data not shown). The SPG concentration was different between inhibition of the macrophage adherence to the plastic plate *in vitro* and reduction of antitumor activity *in vivo*. This might be due to the presence or absence of blood flow and endothelial cell layer.

Leukocyte adhesion deficiency I (LAD I) involves a genetic defect in the biosynthesis of CD18.³⁰ Patients carrying LAD I have frequent and recurrent skin and soft-tissue infections, poor wound healing, and severe periodontal disease. The symptoms are strongly related to the failure of extravascular release of leukocytes. The phenomenon reported here might be similar to the symptoms appearing in LAD I because SPG inhibited adhesion may modulate leukocyte traffic to the tumor.

Intraperitoneally administered SPG gradually migrated to liver and spleen *via* the bloodstream.³¹ During this period, SPG had a chance to interact with various types of cells throughout the body. Carrageenan is known as a blocker of macrophage function and inhibit antitumor activity of BRM. Suda *et al.* found that carrageenan significantly inhibited translocation of (1 $\rightarrow$ 3)- β -D-glucan from the peritoneal cavity.³² We also found that the effect of carrageenan was only shown prior to or at the same time as tumor challenge.³³ A challenge by carrageenan on day 7 after tumor transplantation elicited almost no activity. In contrast, we showed that failure of the antitumor activity by SPG appeared significantly on day 7. The mechanisms of carrageenan and SPG thus would be very different.

SPG has activity to prime mice to produce TNF- α triggered by LPS, and the activity was also reduced by higher concentration of SPG. We have previously shown that SSG also displayed the priming effect and the macrophage expressed higher concentration of TNF- α on the membrane. Higher concentration of SPG might down-regulate the step

involved in this reaction. TNF- α is a pro-inflammatory cytokine and is a marker for bacterial sepsis, which induces systemic inflammatory response syndrome, multiple organ failure, and death. Overexpression of TNF- α is well known to strangle immune system and a higher concentration of SPG might reduce symptoms similar to systemic inflammation.

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