Effect of SCG, 1,3- β -D-Glucan from *Sparassis crispa* on the Hematopoietic Response in Cyclophosphamide Induced Leukopenic Mice

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Sparassis crispa Fr. is an edible mushroom recently cultivable in Japan. It contains a remarkably high content of 6-branched 1,3- β -D-glucan showing antitumor activity. Using ion-exchange chromatography, a purified β -glucan preparation, SCG, was prepared. In this study, we examined the hematopoietic response by SCG in cyclophosphamide (CY)-induced leukopenic mice. SCG enhanced the hematopoietic response in CY induced leukopenic mice by intraperitoneal routes over a wide range of concentrations. SCG enhanced the hematopoietic response in CY-treated mice by prior or post administration. Analyzing the leukocyte population by flow cytometry, monocytes and granulocytes in the peritoneal cavity, liver, spleen and bone marrow (BM) recovered faster than in the control group. The ratio of natural killer cells and $\gamma\delta$ T cells in the liver, spleen and peritoneal cavity was also increased. In contrast, CD4⁺ CD8⁺ cells in the thymus were temporarily significantly decreased by the administration of SCG. Interleukin-6 (IL-6) production of CY+SCG-treated peritoneal exudated cells (PECs), spleen cells and bone marrow cells (BMCs) were higher than that of the CY-treated group. By *in vitro* culture of CY-treated PEC and spleen cells, IL-6 production was enhanced by the addition of SCG. These facts suggested the possibility that IL-6 might be a key cytokine for the enhanced hematopoietic response by SCG.

Key words SCG; β -glucan; hematopoietic response; interleukin-6; natural killer cell

The major side-effect of most anti-cancer chemotherapeutic drugs is neutropenia, and the administration of these drugs impairs blood-forming functions (*e.g.* the generation of neutrophils, NK cells, *etc.*) that are important to maintain the defense systems of the patients. As a result, chemotherapy may accelerate the risk of tumor metastases and fungal infection. Metastasis of tumor cells is a serious concern, causing deterioration in the condition of patients receiving cancer therapy.^{1–3} An immunomodulating substance, a biological response modifier (BRM) or biotherapy is important for the treatment of cancer and infectious diseases. β -Glucan is a well-known BRM which is widely distributed in nature and is used as a medicine and food.^{4–7} Among β -glucans, 6-branched 1,3- β -glucan is the most precisely characterized one. Lentinan from *Lentinus edodes* and Sonifilan (SPG) from *Schizophyllum commune* have been clinically used for cancer therapy in Japan.^{8,9}

Sparassis crispa is an edible mushroom recently cultivable in Japan. Following preliminary investigation, it was found that the β -glucan content of *S. crispa* was 43.6%, as measured by the so-called “enzyme method” by Japan Food Research Laboratories. Thus, *S. crispa* has been found to be a good source material to prepare the antitumor β -glucan in a very high yield. In the previous study, *S. crispa* were repeatedly extracted with hot water (SCHWE), cold NaOH (SCCA), and then hot NaOH (SCHA), and the structure of the polysaccharide fractions of these extracts were analyzed by chemical and enzymic methodologies. The primary structure of the major polysaccharide fraction was found to be 6-branched 1,3- β -glucan, having one branch in approximately every third main chain unit. It shows strong metachromasy to aniline blue, like other gel forming 1,3- β -glucans. All of these fractions showed strong antitumor activity against the solid form of Sarcoma 180 in ICR mice, with strong vascular

dilation and hemorrhage (VDH) reaction by administering a microgram quantity.¹⁰

To examine the pharmacological applicability of *S. crispa*, β -glucan, SCG was purified from SCCA, and its biological activity, especially the effect on cyclophosphamide (CY)-induced leukopenia, was examined in the present study.

MATERIALS AND METHODS

Animals ICR male mice between 6 and 8 weeks of age were purchased from Japan SLC, Shizuoka. Mice were maintained under specific pathogen free conditions.

Preparation of SCG Fruit bodies of *Sparassis crispa* were cultured by Minahealth Co., Ltd., Saitama. SCG was prepared as below: Briefly, air-dried and powdered *S. crispa* (50 g) was defatted with ethanol (4 °C, 2 d) and then extracted with hot water by autoclaving (121 °C, 2 h). The polysaccharide fraction was prepared from the extract by precipitation with 1 vol. and then 4 vol. of ethanol (SCHWE1v; 4.72 g, SCHWE4v; 0.83 g). The resulting residue was then extracted with cold alkali (10% NaOH/5% urea, 4 °C, 2 d, twice; SCCA1 and SCCA2), and hot alkali (10% NaOH/5% urea, 65 °C, 1 h; SCHA). Polysaccharide fractions of alkali extracts were collected after extensive dialysis (SCCA1; 8.97 g, SCCA2; 4.26 g, SCHA; 3.84 g). Residue of the alkaline extract was oxidized by NaClO, then dialyzed and lyophilized. Less than 10% of the total component remained after these extraction steps (SCRE; 3.64 g). SCCA1 dissolved in 8 M urea was applied to a column of DEAE Sephadex A25 (Cl[−]) equilibrated with 8 M urea, and the passed through fraction was collected and dialyzed extensively against tap and distilled water and then was lyophilized (SCG; 73.6%, elemental analysis C:H:N=39.70:6.83:0.09). The SCG solution was prepared by dis-

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solving it in 0.5N NaOH, followed by immediate dialysis against saline for 3 d. After dialysis, the non-dialyzable fraction was autoclaved and frozen until use.

Materials Hank's balanced salt solution (HBSS) was purchased from Nissui Seiyaku Co., Ltd. Tween 20 and Turk's solution were purchased from Wako Pure Chemical Co. Gentamycin sulfate, RPMI 1640 medium and bovine serum albumin (BSA) were from Sigma Chemical Co. Heat inactivated fetal calf serum (FCS) was from Iwaki Co., Chiba. Percoll was from Amersham Pharmacia Biotech, Uppsala, Sweden. CY was from Shionogi & Co., Ltd., Osaka.

Counting of White Blood Cells (WBCs) WBCs were removed from the tail vein of mice injected with CY (200 mg/kg) and SCG (250 μ g/mouse) or from nontreated mice. WBC numbers were counted by mixing blood (5 μ l) with Turk's solution (95 μ l).

Counting of Spleen Cells, Peritoneal Exudate Cells (PECs), Liver Leukocytes, Bone Marrow Cells (BMCs) and Thymocytes Spleen cells, PECs, liver leukocytes, BMCs and thymocytes were removed from mice injected with CY (200 mg/kg) and SCG (250 μ g/mouse) or nontreated mice on 5, 7 or 9 d after administration. The spleen was teased apart in 1 $\times$ PBS. PECs were isolated from the peritoneal cavity by means of lavaging with 10–12 ml HBSS containing 50 μ g/ml gentamycin sulfate. The liver was teased apart in HBSS containing 50 μ g/ml gentamycin sulfate and then purified by 44/70% discontinuous Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation (18 min, 1800 rpm, room temperature). BMCs were obtained by flushing femoral shafts, followed by suspension in 1 $\times$ PBS. The thymus was teased apart in 1 $\times$ PBS. After each centrifugation of spleen cells, PECs, liver leukocytes, BMCs and thymocytes, cells were kept in 1 $\times$ PBS. The leukocyte numbers were counted with each cell suspension in Turk's solution.

Preparation of Spleen Cells, PECs, Liver Leukocytes, BMCs and Thymocytes for FACS Spleen cells, PECs, liver leukocytes, BMCs and thymocytes were removed from mice injected with CY (200 mg/kg) and SCG (250 μ g/mouse) or from nontreated mice. Five, 7 or 9 d after administration, the spleen was teased apart in 1 $\times$ PBS. PECs were isolated from the peritoneal cavity by means of lavaging with 10–12 ml HBSS containing 50 μ g/ml gentamycin sulfate. The liver was teased apart in HBSS containing 50 μ g/ml gentamycin sulfate and then purified by 44/70% discontinuous Percoll gradient centrifugation (18 min, 1800 rpm, room temperature). BMCs were obtained by flushing the femoral shafts, followed by suspension in 1 $\times$ PBS. One, 3, 4, 5, 7 or 9 d after administration, the thymus was teased apart in 1 $\times$ PBS. After each centrifugation of spleen cells, PECs, liver leukocytes, BMCs and thymocytes, cells were maintained in 1 $\times$ PBS supplemented with 0.1% NaN₃ and 2% FCS.

Preparation of Spleen Cells, PECs and BMCs for Cell Culture Spleen cells, PECs and BMCs were removed from mice. The spleen was teased apart in RPMI 1640 medium, and after centrifugation the single cell suspension was treated with an ACK-lysing buffer (8.29 g/l NH₄Cl, 1 g/l KHCO₂, 37.2 mg/l EDTA/2Na) to lyse red blood cells. After centrifugation, spleen cells were maintained in RPMI 1640 medium supplemented with 50 μ g/ml gentamycin sulfate and 10%

FCS. Cells were cultured in 24-well flat bottomed plates at 5×10^6 cells per well in 1 ml of culture medium. PECs were isolated from the peritoneal cavity by lavaging with 10–12 ml HBSS containing 50 μ g/ml gentamycin sulfate. After centrifugation, PECs were maintained in RPMI 1640 medium supplemented with 50 μ g/ml gentamycin sulfate and 10% FCS. Cells were cultured in 24-well flat bottomed plates at 5×10^5 per well in 1 ml of culture medium. BMCs obtained by flushing femoral shafts were suspended in HBSS containing 50 μ g/ml gentamycin sulfate. After centrifugation, BMCs were maintained in RPMI 1640 medium supplemented with 50 μ g/ml gentamycin sulfate containing 10% FCS and cultured in 24-well flat bottomed plates at 1×10^6 per well in 1 ml of culture medium. Spleen cells, PECs and BMCs were cultured at 37 °C for 48 h in a humidified 5% CO₂: 95% air atmosphere.

Flow Cytometry Spleen cells, PECs, liver leukocytes and thymocytes were stained with FITC- and PE-conjugated mAbs. To block FcR-mediated binding of the mAb, anti-mouse CD16/CD32 FcBlock™ (Pharmingen Co., U.S.A.) was added. All incubation steps were performed at 4 °C for 30 min. The cells were analyzed on a FACSCalibur, and data were processed using the CELLQuest program. The following mAbs were used for immunofluorescent staining: PE-anti mouse CD11b (Mac-1) (Pharmingen Co., U.S.A.), FITC-anti mouse Ly-6G (Gr-1) (Pharmingen Co., U.S.A.), PE-anti mouse TCR β chain (Pharmingen Co., U.S.A.), FITC-anti mouse $\gamma\delta$ TCR (Pharmingen Co., U.S.A.), PE-anti mouse CD3- ϵ (Pharmingen Co., U.S.A.), FITC-anti mouse NK-1.1 (Pharmingen Co., U.S.A.), PE-anti mouse CD4 (Pharmingen Co., U.S.A.), FITC-anti mouse CD8 α (Pharmingen Co., U.S.A.). For some experiments, the lymphocyte region was gated by Forward Scatter (FSC) and Side Scatter (SSC) for analysis.

ELISA A 96-well multi-well plate (Sumiron MS-8596F; Sumitomo Bakelite Co., Japan) was coated with capture antibody for rat anti-mouse IL-6 mAb (Pharmingen Co., U.S.A.) in 0.1 M NaHCO₃ buffer (pH 8.2) by overnight incubation at 4 °C. The plate was washed with PBS containing 0.05% Tween 20 (PBST), and blocked with 0.5% BSA (BPBST) at 37 °C for 40 min. After washing, the plate was incubated with rm-IL-6 (Pharmingen Co. U.S.A.) or 50 μ l of test sample at 37 °C for 40 min. The plate was washed with PBST and then treated with antibody for biotinylated rat anti-mouse IL-6 mAb (Pharmingen Co. U.S.A.) in BPBST. Subsequently, the plate was treated with peroxidase conjugated streptavidine (Zymed Laboratories, Inc.) and developed with a TMB substrate system (KPL, Inc., MD, U.S.A.). Color development was stopped with 1 N phosphoric acid and the optical density was measured at 450 nm.

Statistical analysis The results were expressed as the mean $\pm$ standard deviation (SD). The significance of difference between means was measured by Student's *t*-test.

RESULTS

Effect of SCG on White Blood Cell (WBC) Number in CY-Treated Mice The experiment was planned to measure the effects of SCG on CY-induced leukopenia. CY (200 mg/kg) was administered i.p. to ICR mice on day 0. Immediately after CY administration, SCG (125, 250, 500, 1000,

Fig. 1. Effect of SCG Administration on White Blood Cell Number in CY-Induced Leukopenic Mice

CY (200 mg/kg) was i.p. administered to ICR mouse (male, 6 weeks old) on day 0. Immediately after CY administration, SCG (250 [a], 500 [b], 1000 [c] or 2000 µg/mouse [d]) was i.p. administered to these mice. White blood cell number was counted on day 0, 1, 3, 5, 7, 9, 11, 14, 17, 20 and 23 by mixing blood (5 µL, tail vein) with Turk's solution (95 µL). The data shows one of three experiments performed with similar results, each evaluating three mice per group. Values represent mean $\pm$ S.D. Significant difference from CY-treated group, * p < 0.05, ** p < 0.01.

2000 µg/mouse) was administered i.p. to CY-treated mice. The number of WBCs was monitored for 3 weeks. Figure 1 shows the kinetics of WBC count. The number of WBCs was significantly reduced within 3 d after the CY treatment. The peak of the WBC count appeared on day 7 in almost all of the SCG groups. In contrast, it appeared on day 9 in the CY group and the SCG-2000 µg group (Fig. 1). The number of WBCs in the SCG-1000 µg group was significantly higher than that in the CY group from days 5 to 25. Figure 2 shows the dose response of WBC count measured on day 7. The effect of SCG has an optimal dose, and a high concentration of SCG showed low efficacy.

In the next experiment, the timing of administration of SCG in CY-treated mice was changed. Figure 3 shows the kinetics of WBC count. SCG (250 µg/mouse) was adminis-

Fig. 2. Dose Dependence of SCG Administration on White Blood Cell Number in CY-Induced Leukopenic Mice on Day 7

The number of WBC in CY/SCG treated mice on day 7 was plotted against dose. Experimental details are shown in legend of Fig. 1. The data shows one of three experiments performed with similar results, each evaluating three mice per group. Values represent mean $\pm$ S.D. Significant difference from CY-treated group, * p < 0.05, ** p < 0.01.

Fig. 3. Effect of Timing of SCG Administration on White Blood Cell Number in CY-Induced Leukopenic Mice

CY (200 mg/kg) was i.p. administered to ICR mouse (male, 6 weeks old) on day 0. Three or 1 d before (day -3 or day -1) the administration of CY, SCG (250 µg/mouse) was i.p. administered to these mice (a). Immediately after (day 0) or 1 or 3 d after (day 1 or day 3) the administration of CY, SCG (250 µg/mouse) was i.p. administered to these mice (b). Five, 7 or 9 d after (day 5, day 7 or day 9) the administration of CY, SCG (250 µg/mouse) was i.p. administered to these mice (c). White blood cell number was counted by mixing blood with Turk's solution, as shown in Fig. 1. The data shows one of three experiments performed with similar results, each evaluating three mice per group. Values represent mean $\pm$ S.D. Significant difference from CY-treated group, * p < 0.05, ** p < 0.01, *** p < 0.001. (a) $\diamond$: CY (200 mg/kg); $\blacksquare$: day -3; $\blacktriangle$: day -1; $\times$: day 0. (b) $\diamond$: CY (200 mg/kg); $\times$: day 0; $\square$: day 1; $\bullet$: day 3. (c) $\diamond$: CY (200 mg/kg); $\times$: day 0; $\blacktriangle$: day 5; $\bullet$: day 7; $\square$: day 9.

Table 1. Effect of SCG on Leukocyte Number of Lymphoid Organs in CY-Treated Mice

		Leukocyte number ($\times 10^6$)				
		Spleen ^{a)}	PEC ^{a)}	Liver ^{a)}	BM ^{b)}	Thymus ^{a)}
Day 5	CY	73 $\pm$ 32.5	1.6 $\pm$ 1.1	5.9 $\pm$ 1.8	4.3 $\pm$ 1.7	19.5 $\pm$ 4.7
	CY+SCG	185 $\pm$ 91.9	1.3 $\pm$ 0.2	28.9 $\pm$ 23.0	10.6 $\pm$ 0.7	9.4 $\pm$ 2.7
Day 7	CY	178.5 $\pm$ 143	2 $\pm$ 0.9	1.3 $\pm$ 0.7	5.2 $\pm$ 1.7	2.9 $\pm$ 0.5
	CY+SCG	300 $\pm$ 0	4.7 $\pm$ 0.0	38.8 $\pm$ 16	11 $\pm$ 2.7	1.6 $\pm$ 1.2
Day 9	CY	245 $\pm$ 134.4	1.8 $\pm$ 2.3	10.7 $\pm$ 2.8	20.9 $\pm$ 6.5	34.0 $\pm$ 3.8
	CY+SCG	450 $\pm$ 28.3	3.4 $\pm$ 0.6	22.2 $\pm$ 11.6	17.9 $\pm$ 8.3	17.7 $\pm$ 9.1
Normal		231 $\pm$ 1.6	4.7 $\pm$ 0.1	11 $\pm$ 0	5.4 $\pm$ 0.4	26.5 $\pm$ 5.5

The number of leukocytes of Spleen, PEC, Liver, BM or Thymus in CY/SCG treated mice was counted on day 5, 7 or 9 with Turk's solution. The data shows one of three experiments performed with similar results, each evaluating two mice per group. a) per organ. b) per two femoral.

Table 2. Effect of SCG on Macrophages and Granulocytes in CY-Treated Mice

		The number of macrophages or granulocytes ($\times 10^6$)				
		Day 5		Day 7		Normal
		CY	CY+SCG	CY	CY+SCG	
Spleen	Mac-1 ⁺ Gr-1 ⁻	10.2 $\pm$ 0.3	35.9 $\pm$ 18.7	17.8 $\pm$ 0.6	21.7 $\pm$ 1.5	36.3 $\pm$ 7.9
	Mac-1 ⁺ Gr-1 ⁺	4.6 $\pm$ 0.2	57.0 $\pm$ 34.5	69.4 $\pm$ 5.2	119.2 $\pm$ 10.2*	47.4 $\pm$ 14.7
PEC	Mac-1 ⁺ Gr-1 ⁻	0.9 $\pm$ 0.3	1.0 $\pm$ 0.03	1.5 $\pm$ 0.1	1.3 $\pm$ 0.2	4.0 $\pm$ 0.1
	Mac-1 ⁺ Gr-1 ⁺	0.05 $\pm$ 0.02	0.2 $\pm$ 0.01**	0.3 $\pm$ 0.1	1.5 $\pm$ 0.2**	0.05 $\pm$ 0.02
Liver	Mac-1 ⁺ Gr-1 ⁻	0.7 $\pm$ 0.3	14.1 $\pm$ 4.8	0.3 $\pm$ 0.1	7.8 $\pm$ 1.6*	0.4 $\pm$ 0.1
	Mac-1 ⁺ Gr-1 ⁺	0.6 $\pm$ 0.2	6.9 $\pm$ 3.0	0.6 $\pm$ 0.0	23.7 $\pm$ 1.2*	0.4 $\pm$ 0.2
BM	Mac-1 ⁺ Gr-1 ⁻	0.9 $\pm$ 0.1	5.4 $\pm$ 1.4	0.5 $\pm$ 0.1	1.8 $\pm$ 0.4	0.4 $\pm$ 0.2
	Mac-1 ⁺ Gr-1 ⁺	0.9 $\pm$ 0.03	4.0 $\pm$ 0.4*	4.5 $\pm$ 0.2	8.8 $\pm$ 0.3**	4.0 $\pm$ 0.4

Spleen cells, PECs, liver leukocytes and BMCs were isolated from CY/SCG treated mice on day 5 or 7. These cells were stained for Gr-1 and Mac-1, then analyzed by flow cytometry as described in experimental procedures. Mac-1⁺Gr-1⁻ cell; macrophage and Mac-1⁺Gr-1⁺ cell; granulocyte. The data showed total cells $\times$ percentage of macrophages or granulocytes. The data shows one of five experiments performed with similar results, each evaluating two mice per group. Significant difference from CY-treated group, * p <0.05, ** p <0.01.

tered 3 or 1 d before (day -3 or -1) CY-administration (Fig. 3a), immediately after (day 0), or 1 or 3 d after (day 1 or day 3) CY-administration (Fig. 3b) or 5, 7 or 9 d after (day 5, day 7 or day 9) CY-administration (Fig. 3c), and the WBCs were counted. Comparing the kinetics, early administration of SCG (day -1) shifted the peak earlier than that of simultaneous administration. In contrast, later administration of SCG (day 5 or 7) shifted the peak more slowly. These data strongly suggest the enhancement of the hematopoietic response by SCG, and SCG had an optimum dose.

Effect of SCG on Leukocyte Number of Lymphoid Organs in CY-Treated Mice We examined the effect of SCG on leukocyte number in various organs of CY-treated mice. Mice were treated with CY (day 0), and immediately SCG (250 μ g/mouse) was administered i.p. to the CY-treated mice. Leukocytes were isolated from the spleen, PEC, liver, BM and thymus on day 5, 7 or 9, and counted with Turk's solution. The leukocyte number in the spleen was gradually increased in the CY group and SCG group, while that in the SCG group was higher than in the CY group on day 5, 7 and 9 (Table 1). In the liver, the number in the SCG group was higher than in the CY group on days 5, 7 and 9 and in BM, on days 5 and 7. In contrast, in the thymus, the number in the SCG group was lower than in the CY group.

Effect of SCG on Cell Population in CY-Treated Mice To examine the effect of SCG on the cell population in CY-treated mice, surface antigens of leukocytes in various organs were tested by flow cytometry. Mice were treated with CY

(day 0), and immediately SCG (250 μ g/mouse) was administered i.p. to these mice. Leukocytes were isolated from the liver, spleen, PEC, BM and thymus and stained with sets of antibodies: i.e., PE-anti mouse CD11b (Mac-1) and FITC-anti mouse Ly-6G (Gr-1), PE-anti mouse TCR β chain and FITC-anti mouse $\gamma\delta$ TCR, PE-anti mouse CD3- ϵ and FITC-anti mouse NK-1.1, PE-anti mouse CD4 and FITC-anti mouse CD8 α . The administration of SCG increased the number of macrophages (Mac-1⁺Gr-1⁻ cell) and granulocytes (Mac-1⁺Gr-1⁺ cell) in the spleen, PEC, liver and BM on days 5 or 7 more than that of CY (Table 2). Especially, the granulocyte population was increased in the SCG-group on day 5 (PEC - 0.2 $\pm$ 0.01 $\times 10^6$, BM - 4.0 $\pm$ 0.4 $\times 10^6$), and the ratio further increased on day 7 (spleen - 119.2 $\pm$ 10.2 $\times 10^6$, PEC - 1.5 $\pm$ 0.2 $\times 10^6$, liver - 23.7 $\pm$ 1.2 $\times 10^6$, BM - 8.8 $\pm$ 0.3 $\times 10^6$).

The administration of SCG increased the population of NK1.1⁺ cells in PEC and in the spleen on days 5, 7 and 9 more than that of CY (Table 3), especially PEC - day 5 - 10.4 $\pm$ 6.4%, and spleen - day 5 - 3.1 $\pm$ 0.3%. The population of $\gamma\delta$ T cells in the liver, PEC and spleen on day 5 were also increased more than that of CY (Table 4), especially liver - day 5 - 1.8 $\pm$ 0.3%, PEC - day 5 - 10.4 $\pm$ 6.4%, and spleen - day 5 - 3.1 $\pm$ 0.3%.

In the thymus, the population of CD4⁺CD8⁺ double positive thymocytes was reduced on day 3 (Fig. 4). The percentage of CD4⁺CD8⁺ thymocytes per total cells was 25% in CY mice, and 78.3% in normal mice. This increased on

Table 3. Effect of SCG on NK Cells in CY-Treated Mice

		Percentage of CD3 ⁺ /NK1.1 ⁻ or CD3 ⁻ /NK1.1 ⁺ cell for gated cell (%)						
		Day 5		Day 7		Day 9		Normal
		CY	CY+SCG	CY	CY+SCG	CY	CY+SCG	
Liver	CD3 ⁺ /NK1.1 ⁻	41.3±13.1	36.5±6.1	23.6±3.5	22.9±4.2	30.0±6.8	25.4±8.7	33.0±12.1
	CD3 ⁻ /NK1.1 ⁺	2.5±1.0	1.6±0.6	4.3±1.5	1.6±0.5	3.6±1.5	3.7±0.3	4.5±0.7
PEC	CD3 ⁺ /NK1.1 ⁻	19.1±9.0	12.3±2.4	17.7±2.4	16.2±10.0	17.4±4.2	25.0±4.8	15.6±1.1
	CD3 ⁻ /NK1.1 ⁺	1.4±1.0	10.4±6.4*	2.9±1.4	10.8±9.1	2.0±1.0	2.3±0.4	3.6±0.0
Spleen	CD3 ⁺ /NK1.1 ⁻	46.2±2.5	43.4±8.4	12.0±2.6	12.6±3.9	16.4±2.8	13.9±1.3	28.9±4.6
	CD3 ⁻ /NK1.1 ⁺	2.1±0.5	3.1±0.3**	4.4±0.3	4.5±1.6	2.1±0.3	2.1±0.4	3.0±0.7

Liver leukocytes, PECs and spleen cells were isolated from CY/SCG treated mice on day 5, 7 or 9. These cells were stained for CD3 and NK1.1, then analyzed by flow cytometry as described in experimental procedures. The data shows one of three experiments performed with similar results, each evaluating four mice per group. Significant difference from CY-treated group, **p*<0.01, ***p*<0.005.

Table 4. Effect of SCG on $\gamma\delta$ T Cells in CY-Treated Mice

		Percentage of $\alpha\beta^+/\gamma\delta^-$ or $\alpha\beta^-/\gamma\delta^+$ cell for gated cell (%)						
		Day 5		Day 7		Day 9		Normal
		CY	CY+SCG	CY	CY+SCG	CY	CY+SCG	
Liver	$\alpha\beta^+/\gamma\delta^-$	41.4±13.1	36.5±6.1	23.6±3.4	22.9±4.2	30.0±6.8	25.4±8.7	33.0±12.1
	$\alpha\beta^-/\gamma\delta^+$	0.9±0.5	1.8±0.3*	4.3±1.5	1.7±0.5	3.6±1.5	3.7±0.3	4.5±0.7
PEC	$\alpha\beta^+/\gamma\delta^-$	19.1±9.0	12.3±2.4	17.7±2.4	16.2±10	17.4±4.1	25.0±4.8	15.6±1.1
	$\alpha\beta^-/\gamma\delta^+$	1.4±1.0	10.4±6.4*	2.9±1.4	10.8±9.0	1.9±1.0	2.3±0.4	3.6±0.0
Spleen	$\alpha\beta^+/\gamma\delta^-$	46.2±2.4	43.4±8.4	12±2.6	12.6±3.9	16.4±2.8	13.4±1.3	28.9±4.6
	$\alpha\beta^-/\gamma\delta^+$	2.1±0.5	3.1±0.3**	4.4±0.3	4.5±1.6	2.1±0.3	2.1±0.4	3.0±0.7

Liver leukocytes, PECs and spleen cells were isolated from CY/SCG treated mice on day 5, 7 or 9. These cells were stained for TCR- β chain and $\gamma\delta$ TCR, then analyzed by flow cytometry as described in experimental procedures. The data shows one of three experiments performed with similar results, each evaluating four mice per group. Significant difference from CY-treated group, **p*<0.01, ***p*<0.005.

day 4 (70.2% in SCG group, 79.6% in CY group). On day 5 and 7, the administration of SCG increased the population of CD4⁺ or CD8⁺ single positive thymocytes as against CD4⁺ CD8⁺ double positive thymocytes. On day 5, the percentage of CD4⁺ single positive thymocytes for total cells was 30.6%, CD8⁺ single positive thymocytes was 12.4%, and CD4⁺ CD8⁺ double positive thymocytes was 7.0% in the SCG group. On day 9, it recovered to the normal level. These results suggest that the administration of SCG in CY-treated mice significantly modulated the maturation of thymocytes. Taken together, the administration of SCG modified not only cell number but also the population of leukocytes in various organs.

Effect of SCG on IL-6 Synthesis in CY-Treated Mice

IL-6 is a multifunctional hematopoietic cytokine, involved in such processes as B cell differentiation, T cell activation, induction of acute phase proteins, reduction of the G0-residence time of the hematopoietic stem cells, and so on. In order to analyze the enhanced hematopoietic response by SCG, the production of IL-6 by spleen cells, PECs and BMCs were examined. Mice were treated with CY (day 0), and immediately SCG (250 μ g/mouse) was administered i.p. to these mice. Spleen cells, PECs or BMCs were prepared on days 5, 7 and 9, and cultured *in vitro* to collect the supernatant. The concentration of IL-6 was measured by ELISA. As shown in Fig. 5, IL-6 concentration in spleen cells and PEC culture was significantly increased in SCG administered mice on day 5. The concentration in BMC culture was significantly increased in SCG administered mice on day 7. And that in spleen cells, PECs and BMC culture declined in those

mice administered SCG on day 9. These results suggest that IL-6 might be a key to inducing the hematopoietic response of SCG.

Effect of SCG on IL-6 Synthesis in CY-Treated Mice *in Vitro* To analyze the effect of SCG on IL-6 synthesis of leukocytes, the production of IL-6 by spleen cells and PECs were examined *in vitro*. Mice were treated with CY or saline (day 0). Spleen cells and PECs were prepared on days 1—9 and cultured with saline or SCG (100 μ g/ml) *in vitro* to collect the supernatant. The concentration of IL-6 was measured by ELISA. As shown in Fig. 6, the IL-6 concentration in spleen cells and PEC culture was increased in CY mice (day 1 or 3) by SCG. In contrast, spleen cells and PECs prepared on days 5 and 7 produced a significantly higher concentration of IL-6 in response to SCG. These results suggest that the number of cells responsible for SCG was increased in CY-treated mice, especially during days 5—7.

DISCUSSION

We have long been working on the relationship between structure and immunomodulating activity of β -glucans, using *G. frondosa*,^{11,12)} *P. vesiculosa*,¹³⁾ *S. sclerotiorum*,^{14,15)} *S. commune*,¹⁶⁾ *O. lapidescens*,¹⁷⁾ *G. lucidum*,¹⁸⁾ *S. cerevisiae*,¹⁹⁾ *C. albicans*,²⁰⁾ and *M. furfur*.²¹⁾ During this period, we found the activity was significantly dependent on the molecular weight, degree of branching, and conformation, in a preclinical animal study.^{22,23)} Cancer immunotherapy has been applied to many patients, and enhancement of the quality of life of patients is also an important goal of cancer treatment. Estab-

Fig. 4. Effect of SCG on Thymocyte Population in CY-Treated Mice

Thymocytes were isolated from CY/SCG treated mice on day 1, 3, 4, 5, 7 or 9. These cells were stained for CD4 and CD8, then analyzed by flow cytometry, as described in Experimental procedures. The data shows one of five experiments performed with similar results, each evaluating two mice per each group.

lishment of a molecular mechanism for use in biotherapy is still needed. *S. crispa* is a mushroom recently cultivable in Japan, and was found to be a good source material to prepare antitumor β -glucan in a very high yield. The structure of the main component was 6-branched 1,3- β -glucan having branches approximately one in every third main chain. We previously reported that all fractions of *S. crispa* showed antitumor activity to the solid form of Sarcoma 180 in ICR mice, with strong vascular dilation and hemorrhage reaction and enhanced hematopoietic response to CY induced leukopenic mice following intraperitoneal or oral administration.¹⁰⁾ The present study confirmed and expanded the usefulness of *S. crispa* in cancer immunotherapy.

Reconstruction of the hematopoietic response is an important event in advanced medical treatment, such as chemotherapy and/or gamma irradiation to the advanced cancer patients and in treating hypoplastic anemia. Recently, techniques to use the hematopoietic progenitors in peripheral blood or in cord blood have been significantly improved by developing applications of cytokines, their genes or their inducers. It is believed that β -glucans show various immunomodulating activity with low toxicity both in experimental animals and in the clinic.²⁴⁻²⁸⁾ In the present study, we showed an enhanced hematopoietic response in CY-induced leukopenic mice both from a qualitative as well as quantitative point of view. The data shown here might give an additional proof of efficacy of β -glucan as a candidate for these medical treatments.

In the present study, we detected a high concentration of IL-6 *in vivo* and *in vitro*. In the previous study using a clinically applied β -glucan, sonifilan, we identified significantly high concentrations of IL-6 in bone marrow, spleen, and serum of CY and sonifilan treated mice. Recently, Tritarelli *et al.*²⁹⁾ reported that IL-6 had a strong hematopoietic response in CY-treated mice in the presence of an additional cytokine, IL-1. It is also suggested that the typical hematopoietic factors are not necessarily required to increase

Fig. 5. Effect of SCG Administration on IL-6 Synthesis in CY-Treated Mice

Spleen cells, PECs or BMCs were isolated from CY/SCG treated mice on day 5, 7 or 9. Cell suspensions were adjusted to 5×10^6 , 5×10^5 or 1×10^6 in RPMI medium supplemented with gentamycin sulfate ($5 \mu\text{g}/\text{mL}$) containing 10% heat inactivated fetal calf serum (FCS) and were incubated for 48 h. After incubation, the supernatant was collected. IL-6 concentration in the supernatant was determined by ELISA. The data shows one of four experiments performed with similar results, each evaluating two mice per group. Values represent mean $\pm$ S.D. Significant difference from CY-treated group, * $p < 0.05$, ** $p < 0.01$.

Fig. 6. Effect of SCG Administration on IL-6 Synthesis in CY-Treated Mice *in Vitro*

CY (200 mg/kg) or saline was i.p. administered to ICR mouse on day 0. Spleen cells or PECs were isolated on day 1, 3, 5, 7 or 9. Cell suspensions were adjusted to 5×10^6 or 5×10^5 in RPMI medium supplemented with gentamycin sulfate (5 μ g/ml) containing 10% heat inactivated fetal calf serum (FCS), and were incubated for 48 h with SCG (100 μ g/ml) or saline. After incubation, the supernatant was collected. IL-6 concentration in the supernatant was determined by ELISA. The data shows one of four experiments performed with similar results, each evaluating two mice per group. Values represent mean $\pm$ S.D. Significant difference from control, * $p < 0.05$. (a) days 1, 3; (b) days 5, 7, 9.

the hematopoietic response. In addition, by *in vivo* and *in vitro* cytokine mRNA mapping, the cytokines directly induced by sonifilan in bone marrow cells were M-CSF and stem cell factor (SCF), thus, IL-6 would be induced by following autocrine and/or paracrine signaling concomitant with the growth and differentiation of immature cells, including stem cells.³⁰ Like other cytokines, IL-6 has a variety of biological activities, such as B cell differentiation, T cell activation, induction of acute phase proteins, reduction of the G0-residence time of the hematopoietic stem cells, and so on.³¹ At least a part of the hematopoietic activity of SCG shown in this study would be explainable by IL-6. It is also suggested that CY or the active metabolite, nitrogen mustard, also induces cytokines *in vivo* and *in vitro*.^{32–34} We have

also identified the enhanced expression of IL-1 α , IL-6, M-CSF, and SCF mRNA by CY-administration.³⁰ These facts strongly suggest that multiple cytokines concomitantly increased by CY and SCG would be the underlying mechanism.

Using the immunochemical characterization of leukocyte populations by flow cytometry, in addition to granulocytes and monocytes, other leukocyte populations were found to be modulated by SCG administration, *i.e.*, $\gamma\delta$ T cells and NK1.1 cells. Maturation of thymocyte was also modulated. $\gamma\delta$ T and NK cell populations function especially in the innate and early induced response, which is critically important for establishing the acquired immunity,^{35–39} *i.e.*, NK cells preferentially lyse virus-infected cells and transformed cells by

their cytotoxic function, and $\gamma\delta$ T lymphocytes have been shown to regulate the immune response in diverse experimental systems. The thymus is known to be atrophied by CY-administration due to apoptosis of thymocytes.^{40–42)} Hiramane *et al.*⁴¹⁾ showed a marked decrease in the proportion of CD4⁺CD8⁺ subset in the thymus of mice on day 2 after CY treatment, while the CD4⁺CD8⁺ subset rebounded on day 7. In the present study, CD4⁺CD8⁺ double positive thymocytes of CY-treated as well as CY+SCG treated mice were significantly decreased on day 3 and rebounded on day 7. However, CD4⁺ and CD8⁺ single positive thymocytes, especially on day 4 and day 7, were increased faster by the administration of SCG (Fig. 4). It is well documented that thymocytes are matured from CD4⁺CD8⁺ cells to single positive CD4⁺ or CD8⁺ cells. The higher ratio of these cells in CY+SCG treated mice suggested that SCG might contribute to the maturation of thymocytes.

In the *in vitro* cell culture, we demonstrated strong cytokine productivity of SCG in the spleen and PEC of CY-treated mice. The response could be related to the number and/or the property of SCG-responding cell populations. Soluble β -glucans are generally a rather weak stimulator of cytokines *in vitro* although they showed strong immunopharmacological effects *in vivo*. In the previous study, we showed that cytokine production of macrophages *in vitro* by β -glucans were significantly augmented by the co-existence of IFN- γ .¹⁶⁾ Additionally, macrophage and granulocyte numbers were significantly augmented in CY-treated mice. Taken together, monocyte/macrophage lineage cells might be a candidate of SCG-responding cells in CY-treated mice.

As shown above, the cytokine productivity of CY-administered mice was enhanced spontaneously, thus the autocrine and/or the paracrine mechanisms might have an influence on the cytokine productivity of SCG *in vitro*. Compared with recombinant cytokines, traditional BRMs are usually "cytokine inducers", and the duration of cytokines produced after BRM treatment is significantly longer. This difference in pharmacokinetics would be advantageous in the traditional BRM, including *S. crispa*. As shown in Fig. 3, enhancement of the hematopoietic response was shown by SCG administration prior (day -1 or -3) or after (day 1, 3, 5 or 7) the CY-administration. As shown above, the SCG-responding cell population was increased in the spleen and PEC by CY-administration, thus, the mechanism of the latter administration schedule would be related to the newly developed populations. In contrast, the mechanism of the former administration schedule might be related to the different underlying events.

During analysis of immunopharmacological mechanisms, it is confirmed that 1,3- β -glucans activate macrophages to produce various mediators, even in normal mice.^{43–45)} In addition, SCG induced splenomegaly and VDH (vascular dilation and hemorrhage) reactions in normal mice. These facts suggest that SCG induces hematopoietic response in normal mice. Patchen *et al.*⁴⁶⁾ reported that the administration of a yeast β -glucan preparation, PGG-glucan, enhanced the production of peripheral blood progenitor cells (PBPC) in mice. It is also well documented that B cells are more susceptible to CY-treatment than T cells.⁴⁷⁾ Pre-treatment of IL-1 or IL-6 has enhanced the recovery of bone marrow transplant patients, because of increasing numbers of CY-resistant progen-

itors.^{48–50)} Considering these facts, modulation of the hematopoietic response with prior SCG-administration in CY-treated mice would be related to the enlargement of CY-resistant progenitor populations in mice.

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