Enhancement of Hematopoietic Response of Mice by Intraperitoneal Administration of a β -Glucan, SSG, Obtained from *Sclerotinia sclerotiorum*

Koichi HASHIMOTO, Iwao SUZUKI,* Masumi OHSAWA, Shozo OIKAWA,** and Toshiro YADOMAE*

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

(Received March 15, 1990)

Effects of intraperitoneal (i.p.) administration of SSG, a (1 $\rightarrow$ 3)- β -D-glucan obtained from the culture filtrate of *Sclerotinia sclerotiorum* IFO 9395, on hematopoietic responses of mice were investigated. Numbers of spleen and peripheral leukocytes increased 7 d after administration of SSG (250 μ g/mouse). The percentages of polymorphonuclear leukocytes (PMN) in both spleen and peripheral blood increased markedly. The numbers of macrophage progenitor cells also increased in both spleens and femurs of the mice administered SSG. Furthermore, levels of colony-stimulating activity (CSA) in sera were elevated, and two peaks were observed (at 6 h and on day 7 after administration). Elevated CSAs were also observed in the cultures of peritoneal cells taken from mice 3 or 6 h after administration of SSG. These results may suggest that i.p. administration of SSG into mice enhanced the production of colony-stimulating factors (CSFs), and then increased the numbers of both spleen and peripheral blood leukocytes.

Keywords — (1 $\rightarrow$ 3)- β -D-glucan (SSG); colony-stimulating activity; hematopoietic response; *Sclerotinia sclerotiorum*

Introduction

The major side-effect of most anti-cancer chemotherapeutic drugs is neutropenia, and limits both the doses of drugs used and the frequencies of treatment.¹⁾ Myelosuppression such as neutropenia causes sepsis which results in deaths of most cancer patients by microbial infection.^{2,3)} Therefore, the development of agents capable of enhancing the proliferation of granulocytes and macrophages has been emphasized. Recently, colony-stimulating factors (CSFs) which control the proliferation of hematopoietic cells were purified, and the recombinant proteins were obtained.^{4–8)}

In a previous paper, we reported that an antitumor (1 $\rightarrow$ 3)- β -D-glucan, SSG, was isolated from the culture filtrate of *Sclerotinia sclerotiorum* IFO 9395.⁹⁾ SSG has a different chemical structure and biological activities from the other (1 $\rightarrow$ 3)- β -D-glucans such as SPG, lentinan and grifolan, as follows: (1) The number of branching points in the glucosyl chain of SSG is high.⁹⁾ SSG branches at every two main chain glucosyl units at position C-6. Almost all (1 $\rightarrow$ 3)- β -D-glucans possess fewer branching points. For example, the ratio of branching points to main

chain glucosyl units in SPG and lentinan is 1 to 3 and 2 to 5, respectively;^{10–12)} and (2) SSG shows immunomodulating functions when it is administered by oral route¹³⁾ as well as parenteral routes.¹⁴⁾

In this study, we examined the effects of i.p. administered SSG on the induction of colony-stimulating activity (CSA) and hematopoietic responses in mice.

Material and Methods

Mice — Male ICR mice were obtained from Japan SLC, Inc., Shizuoka. Mice used were 6–10 weeks of age and placed under specific pathogen free conditions.

Preparation of SSG — *S. sclerotiorum* IFO 9395 was grown in the medium containing glucose (2%), yeast extract (0.3%) and polypeptone (1%) with reciprocal shaking at 27 °C for 5 d or upon termination of gas formation. The mycelia and the broth were separated by filtration with a glass filter. The filtrate was combined with 1 volume of ethanol, and the fibrous product which rose to the surface was collected. This material was reprecipitated from aqueous solution by the addition of ethanol (1 volume).

After three ethanol precipitations, the product was dried with acetone and ether. The material was dissolved in 8 M urea and applied to a column of DEAE-Sephadex A-25 (Cl⁻ form) and eluted with 8 M urea. The eluate was dialyzed against tap water and then distilled water, and concentrated. The addition of ethanol (1 volume) yielded SSG which contained less than 1% of protein and was >98% carbohydrate.

Determination of Number and Population of Leukocytes — The suspension of single spleen cells obtained by teasing in Hank's balanced salt solution (HBSS; Nissui Seiyaku Co., Ltd., Tokyo) and blood were diluted in Turk's solution, and the total number of leukocytes per mm³ was counted in a hemocytometer. Smears of spleen cells and blood were stained with May-Grünwald-Giemsa, and the numbers of lymphocytes, polymorphonuclear leukocytes (PMN) and monocytes-macrophages were counted.

Preparation of the Sources of CSA from Mice — Sera were obtained from mice injected with SSG or untreated mice, and were pooled (three mice/group), and then filtered through a membrane filter (pore size; 0.2 μ m). Peritoneal cells from mice treated with SSG were recovered by lavage of the peritoneal cavity with HBSS. These cells were washed twice with Dulbecco's modified Eagle medium (DMEM; Grand Island Biological Co., Grand Island, NY), suspended

at 1×10^6 /ml in DMEM supplemented with 10% fetal calf serum (FCS; Boehringer Mannheim, GmbH) and antibiotics. Aliquots (1 ml) of the cell suspension were placed in a flat-bottomed 24-well tissue culture plate (Sumitomo Bakelite Co., Ltd., Tokyo) and incubated at 37 °C in an atmosphere of 5% CO₂ and air for 40 h. After incubation, the culture medium was harvested and centrifuged at $180 \times g$ for 10 min, and the supernatants were filtered through a membrane filter (0.2 μ m).

Determination of CSA — CSA was determined by using a single layer methylcellulose system. Briefly, bone marrow cells (5×10^4 or 1×10^5) obtained by flushing femoral shafts were suspended in 1 ml of DMEM containing 20% FCS, penicillin G (100 U/ml), streptomycin (100 μ g/ml), 0.88% methylcellulose and 5% sera or 20% culture supernatants of peritoneal cells. The cell suspension was plated in a 35-mm-diameter plastic dish (Nunc, Roskilde). Seven days after incubation, cellular aggregates with more than 50 cells were scored as colonies.

Determination of Macrophage Colony-Forming Cells (CFUm) — L-cell conditioned medium (LCM) was prepared as follows. L 929 fibroblasts were cultured at 1×10^6 cells in 10 ml of RPMI 1640 (Nissui) containing 10% FCS and antibiotics for 5 d. The culture medium was centrifuged at $180 \times g$ for 10 min, and then the

TABLE I. Kinetics of Cell Number and Population in Spleen and Peripheral Blood Leukocytes of Mice Injected i.p. with SSG^{a)}

Days	Cell number	Cell population (%)		
		Lymphocytes	PMN	Macrophages/ monocytes
Spleen ($\times 10^7$ /mouse)				
Untreated	13.4 $\pm$ 0.7	90.5 $\pm$ 2.0	4.4 $\pm$ 1.1	5.2 $\pm$ 1.7
3	11.6 $\pm$ 0.7 ^{b)}	83.4 $\pm$ 5.1	10.4 $\pm$ 2.7 ^{b)}	6.3 $\pm$ 2.4
7	22.1 $\pm$ 2.1 ^{c)}	78.4 $\pm$ 1.5 ^{c)}	12.2 $\pm$ 0.3 ^{d)}	9.4 $\pm$ 1.2 ^{b)}
14	15.3 $\pm$ 0.8	88.6 $\pm$ 3.5	6.9 $\pm$ 1.2	4.6 $\pm$ 2.2
Blood ($\times 10^3$ /mm ³)				
Untreated	11.3 $\pm$ 4.4	71.1 $\pm$ 3.1	25.7 $\pm$ 4.2	3.3 $\pm$ 1.9
3	16.3 $\pm$ 1.3	61.5 $\pm$ 7.8	32.0 $\pm$ 7.0	6.6 $\pm$ 0.8
7	25.2 $\pm$ 3.3 ^{b)}	46.2 $\pm$ 17.5	50.5 $\pm$ 15.8	3.3 $\pm$ 1.8
14	5.0 $\pm$ 2.2	30.7 $\pm$ 11.3 ^{c)}	61.7 $\pm$ 15.7 ^{b)}	7.6 $\pm$ 4.4

a) SSG (250 μ g) was administered i.p. to ICR mice on day 0. Results are expressed as arithmetic mean $\pm$ S.D. of three ICR mice per group. b) $p < 0.05$. c) $p < 0.01$. d) $p < 0.001$.

supernatant was filtered through a membrane filter (0.2 μ m). The number of macrophage progenitor cells that would proliferate in response to LCM was determined by using a single layer methylcellulose system. Briefly, spleen (1×10^6) or bone marrow (1×10^5) cells were suspended in 1 ml DMEM supplemented with 20% FCS, antibiotics, 0.88% methylcellulose and 10% LCM, and then the cell suspension was plated in a 35-mm-diameter plastic dish (Nunc). Seven days after incubation, cellular aggregates with more than 50 cells were scored as colonies.

Statistics — All results are expressed as the mean $\pm$ standard deviation (S.D.). The significance of difference between means was determined by Student's *t*-test.

Results

Changes in Numbers and Populations of Spleen and Peripheral Blood Leukocytes after i.p. Administration of SSG

We first examined the effects of i.p. administration of SSG into mice (250 μ g) on the numbers and populations of spleen and peripheral

blood leukocytes (Table I). Numbers of both spleen and peripheral blood leukocytes increased significantly 7 d after administration of SSG. In spleen cells, percentages of both PMN and macrophages were increased by administration of SSG, and reached a maximum on day 7. In peripheral blood leukocytes, percentages of PMN increased markedly dependent on the number of days after administration of SSG.

Changes in Numbers of CFUm in Spleen and Femur after Administration of SSG

Changes in the number of monocyte-macrophage progenitor cells (CFUm) after administration of SSG were examined by using the cultures of spleen and femur cells with LCM (Fig. 1). The number of CFUm in the spleen began to increase on day 4, and reached a maximum (6 times of control group). On the other hand, the number of CFUm in the femur increased slightly on day 6.

Changes in Serum CSA after Administration of SSG

In the first experiment, mice were administered SSG i.p., and their sera were obtained 3, 6, 12, 18 and 24 h after the administration.

Fig. 1. Changes in Number of CFUm in Spleen and Femur after Administration of SSG

SSG (250 μ g) was administered i.p. into ICR mice. Spleen and bone marrow cells obtained from these mice were harvested on the designated days. Spleen (1×10^6) or bone marrow (1×10^5) cells were cultured in the presence of 10% LCM as exogenous CSF. After 7 d of culture, colony numbers were counted. Each point represents the mean and S.D. of three mice. Symbols: $\circ$, bone marrow; $\bullet$, spleen.

Fig. 2. Changes in Serum CSA 0—24 h after Administration of SSG

SSG (250 μ g) was administered i.p. into ICR mice. Sera were obtained from three mice in one group on the designated hours and pooled. Normal bone marrow cells (5×10^4) were incubated in the presence of 5% these sera. After 7 d of culture, colony numbers were counted. Each point represents the mean and S.D. of triplicate dishes.

Fig. 3. Changes in Serum CSA 3, 7, and 14 d after Administration of SSG

SSG (250 μ g) was administered i.p. into ICR mice on day 0. Sera were obtained from three mice in one group on day 3, 7, and 14 and pooled. Normal bone marrow cells (1×10^5) were incubated in the presence of 5% these sera. After 7 d of culture, colony numbers were counted. Each point represents the mean and S.D. of triplicate dishes.

Fig. 4. Generation of CSA in the Culture Supernatant of the Peritoneal Cells of the Mice Administered SSG

Peritoneal cells were obtained from mice treated with SSG (250 μ g) 0–24 h after administration and cultured at a concentration of 1×10^6 cells/ml. After 40 h of culture, these culture supernatants were harvested. And then, normal bone marrow cells (1×10^5) were incubated in the presence of 20% of these culture supernatants. After 7 d of culture, colony numbers were counted. Each point represents the mean and S.D. of three mice.

As shown in Fig. 2, serum CSA began to rise at 3 h, reached a maximum at 6 h, and the activity decreased to control level at 24 h. Next, CSAs of sera obtained 3, 7 and 14 d after SSG injection were determined. As the result, CSA of serum collected 7 d after administration was higher than that of control mice (Fig. 3).

Changes in CSA of Culture Supernatant of Peritoneal Cells after Administration of SSG

In order to examine whether the administration of SSG affects the CSAs in cells located around the injection site, the culture supernatants of peritoneal cells from SSG i.p. injected mice were determined. As shown in Fig. 4, the increment of CSA was observed in the culture supernatant of peritoneal cells collected 3 and 6 h after administration of SSG.

Discussion

Intraperitoneal administration of SSG increased the number of peripheral blood leukocytes, especially PMN and monocyte-macrophages as well as spleen leukocytes (Table I). Numbers of CFUm in both spleen and femur were also increased by i.p. administration of SSG (Fig. 1). Furthermore, we found that elevated CSA in the sera of mice showed two peaks at 6 h and on day 7 (Figs. 2, 3). These observations may indicate that CSFs produced by i.p. administration of SSG act on macrophage and neutrophil precursor cells in both femur and spleen, and caused an increment in the numbers of macrophages and PMN in both spleen and peripheral blood.

CSAs in culture supernatants of peritoneal cells increased in both 3 and 6 h after administration of SSG as well as in sera (Fig. 4). Several possibilities may be considered to explain why SSG administered i.p. elevated CSA in sera, as follows; 1) CSF produced by peritoneal cells was transferred to peripheral blood, 2) SSG itself was transferred to blood and enhanced CSFs production of peripheral blood leukocytes, and 3) the activated peritoneal cells producing CSFs were transferred to blood. In order to elucidate these problems, detailed examinations are in progress.

We have previously reported that the i.p. administration of SSG activated the antitumor ef-

fector cells such as macrophages, natural killer cells, cytotoxic T lymphocytes, and showed antitumor activities against syngenic tumor cells.¹⁴⁾ Grabstein *et al.* reported that granulocyte-macrophage colony-stimulating factor (GM-CSF) could induce the tumoricidal activity of macrophages.¹⁵⁾ Furthermore, Shimizu *et al.* reported that mature macrophages possessing strong antitumor activities *in vivo* and *in vitro* were induced by the culturing of bone marrow cells for 7 d with the CSA induced by a Gram positive bacterium *Lactobacillus casei* YIT 9018 (LC 9018).¹⁶⁾ It may be that CSA augmented by the injection of SSG induces tumoricidal macrophages, and participates in the development of antitumor activity of SSG.

A lot of biological response modifiers (BRMs) have been studied as anti-cancer drugs, and some of them, OK-432,¹⁷⁾ PSK,¹⁸⁾ lentinan,¹¹⁾ SPG¹⁰⁾ and bestatin,¹⁹⁾ have been applied clinically in Japan. However, the regression of tumor in patients by the use of BRM alone is considered difficult.²⁰⁾ The validity of these BRMs is in the prevention of myelosuppression which is the major side-effect of most chemotherapeutic drugs. Therefore, BRMs possessing an ability to enhance hematopoietic responses are very useful in chemotherapy of cancer. We will hereafter examine the effect of SSG on hematopoietic responses in mice treated with chemotherapeutic drugs.

Acknowledgments We express our thanks to Mr. A. Kinoshita, Mr. T. Sakurai, Miss M. Konishi, Mr. M. Suda and Miss R. Aoki for their technical assistance.

References

- 1) J. P. Okunewick and J. C. Marsh (eds.): Proceeding of symposium on myelosuppressive effects of antineoplastic drugs, *Exp. Hematol*, **13** (suppl 16), 1—122 (1985).
- 2) C. A. De Jongh and S. C. Schimpff: Bacterial infections in neutropenic patients in supportive care, "Cancer Therapy," ed. by D. J. Higby Dordrecht: Martinus Nijhoff, 1983, pp. 1—14.
- 3) T. Fujimoto: Antibiotic therapy acute infection in patients with cancer, *Jpn. J. Cancer Chemother.*, **13**, 2914—2921 (1986).
- 4) H. Nomura, I. Imazeki, M. Oheda, N. Kubota, M. Tamura, M. Ono, Y. Ueyama and S. Asano: Purification and characterization of human granulocyte colony-stimulating factor (G-CSF), *EMBO J.*, **5**, 871—876 (1986).
- 5) L. M. Souza, T. C. Boone, J. Gabilove, P. H. Lai, K. M. Zsebo, D. C. Murdock, V. R. Chazin, J. Bruszewski, H. Lu, K. K. Chen, J. Barendt, E. Platzer, M. A. S. Moore, R. Mertelsmann and K. Welte: Recombinant human granulocyte colony-stimulating factor: Effects on normal and leukemic myeloid cells, *Science*, **232**, 61—65 (1986).
- 6) G. G. Wong, J. S. Witek, P. A. Temple, K. M. Wilkens, A. C. Leary, S. S. Jones, E. L. Brown, R. M. Kay, E. C. Orr, C. Shoemaker, D. W. Golde, R. J. Kaufman, R. M. Hewick, E. A. Wang and S. C. Clark: Human GM-CSF: Molecular cloning of the complementary DNA and purification of the natural and recombinant proteins, *Science*, **228**, 810—815 (1985).
- 7) E. S. Kawasaki, M. B. Ladner, A. M. Wang, J. V. Arsdell, M. K. Warren, H. Y. Coyne, V. L. Schweickart, M. T. Lee, K. J. Wilson, A. Boosman, E. R. Stanley, P. Ralph and D. F. Mark: Molecular cloning of a complementary DNA encoding human macrophage-specific colony-stimulating factor (CSF-1), *Science*, **230**, 291—296 (1985).
- 8) Y. C. Yang, A. B. Ciarletta, P. A. Temple, M. P. Chung, S. Covacic, J. S. Witek-Giannotty, A. C. Jeary, R. Kriz, R. E. Donakue and G. G. Wong: Human IL-3 (multi-CSF): Identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3, *Cell*, **47**, 3—10 (1986).
- 9) N. Ohno, I. Suzuki and T. Yadomae: Structure and antitumor activity of a β -1, 3-glucan isolated from the culture filtrate of *Sclerotinia sclerotiorum* IFO 9395, *Chem. Pharm. Bull.*, **34**, 1362—1365 (1986).
- 10) I. Azuma: Development of immunostimulants in Japan, "Immunostimulants Now and Tomorrow," ed. by I. Azuma and G. Jolles, Japan Scientific Societies Press, Tokyo, 1987, pp. 41—56.
- 11) S. Fujimoto, K. Orita, T. Kondoh, T. Taguchi, K. Yoshida, T. Kimura, N. Ogawa and H. Furue: Modern trends in immunomodulators: Sizofiran, *Biotherapy*, **2**, 500—508 (1988).
- 12) T. Taguchi, Y. Kaneko and G. Chihara: Lentinan, *Biotherapy*, **2**, 509—521 (1988).
- 13) I. Suzuki, K. Hashimoto, N. Ohno, H. Tanaka and T. Yadomae: Immunomodulation by orally administered β -glucan in mice, *Int. J. Immunopharmac.*, **11**, 761—769 (1989).
- 14) 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 syngenic tumors in mice, *J. Pharmacobio-Dyn.*, **11**, 527—532 (1988).
- 15) K. H. Grabstein, D. L. Urdal, R. J. Tushinski, D. Y. Mochizuki, V. L. Price, M. A. Cantrell, S. Gillis and P. J. Conlon: Induction of macrophage tumoricidal activity by granulocyte-macrophage colony-stimulating factor, *Science*, **232**, 506—508 (1986).

- 16) T. Shimizu, K. Nomoto, T. Yokokura and M. Mutai: Role of colony-stimulating activity in antitumor activity of *Lactobacillus casei* in mice, *J. Leuk. Biol.*, **42**, 204—212 (1987).
- 17) T. Hoshino and A. Uchida: OK-432. Recent investigations on its effective mechanisms and optimal therapeutic protocol, *Biotherapy*, **2**, 486—495 (1988).
- 18) K. Ogoshi and T. Mitomi: PSK, *Biotherapy*, **2**, 496—499 (1988).
- 19) S. Tukagoshi: A new antitumor drug with immunomodulating activity, ubenimex (bestatin), *Jpn. J. Cancer Chemother*, **14**, 2385—2391 (1987).
- 20) H. Furue: Immunostimulators, *Biotherapy*, **1**, 25—30 (1987).