Intravenously Administered (1→3)-β-D-Glucan, SSG, Obtained from *Sclerotinia sclerotiorum* IFO 9395 Augments Murine Peritoneal Macrophage Functions *in Vivo*

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Effect of intravenously (i.v.) or intraperitoneally (i.p.) administered (1→3)-β-D-glucan, SSG, obtained from *Sclerotinia sclerotiorum* IFO 9395 on the murine peritoneal macrophage (PM) functions were examined. A single i.v. administration of SSG increased the number of PMs at a dose of 250 μg/mouse, and the peak appeared 4 d after administration. However, no special change was observed on peritoneal exude cell (PEC) populations. These PMs showed augmented lysosomal enzyme activity and the peaks appeared in 2 phases, on days 2 and 10. In contrast, SSG administered i.p. (250 μg/mouse) increased the number of PMs and enhanced the lysosomal enzyme activity of PMs from day 4, and a broad peak appeared until days 8—12. The populations of PECs were also changed by i.p. injection of SSG. Additionally, SSG administered i.v. enhanced phagocytic activity, H_2O_2 production and interleukin 1 (IL-1) production, and the kinetics of the activation differed depending on the activities. These data suggest that the effects of SSG on macrophage functions are different depending on administration routes, and there are some different mechanisms in the activation of macrophages *in vivo* by SSG.

Keywords (1→3)-β-D-glucan; SSG; *Sclerotinia sclerotiorum* IFO 9395; peritoneal macrophage; intravenous administration

Introduction

β-Glucans with 1, 3 and/or 1, 6 linkages are ubiquitous in nature as the major structural components of yeasts and fungi,[1] and are well known to possess immunomodulating effects and exhibit significant antitumor activities.[2] Clinically, two kinds of (1→3)-β-D-glucans, lentinan (from *Lentinus edodes*) and schizophyllan (from *Schizophyllum commune*), have been applied as biological response modifiers (BRMs) in Japan.[3,4] Recent studies by our research group suggested that the biological effects of (1→3)-β-D-glucans were significantly affected by their chemical properties, such as side chain, molecular weight and ultrastructure.[5-7] Therefore, we think that not all of the biological activities of (1→3)-β-D-glucans have still not been necessarily clarified yet. We have been studying the immunomodulating and antitumor activities of a soluble highly branched (1→3)-β-D-glucan, SSG, obtained from the liquid-cultured filtrate of the fungus *Sclerotinia sclerotiorum* IFO 9395 belonging to Ascomycotina.[8-17] Characteristics of the chemical and physical properties of SSG is as follows: (1) SSG branches at every other main chain glucosyl unit at position C-6[8]; (2) SSG has an average molecular weight of $>5\times10^6$; and (3) the viscosity of SSG is high in comparison with the other (1→3)-β-D-glucans.[9] SSG is effective on mice when administered parenterally as well as orally.[12,13,16,17]

Macrophages are known to play a central role in the regulation of specific and nonspecific immunity.[18,19] There have also been reports that macrophages play an important role in the immunomodulating and antitumor effects of BRMs, including (1→3)-β-D-glucans.[4,10,20] The macrophage activation *in vivo* by BRMs differed depending on administration routes.[21,22] It was described previously that (1→3)-β-D-glucans exhibit the strongest immunomodulating effect when administered intravenously (i.v.),[23] and one of the (1→3)-β-D-glucans, lentinan, was administered i.v. to cancer patients clinically.[3] SSG also exhibited the most effective antitumor activities when administered i.v. in mice.[10,11] Therefore, the investigations on macrophage functions after i.v. administration of SSG would be of great value in enhancing our understanding of potential applications.

In this study, we examined the effects of i.v. SSG administration on murine peritoneal macrophage (PM) functions (lysosomal enzyme activity, phagocytic activity, H_2O_2 production, interleukin 1 (IL-1) production, increment of PM number), including detailed examinations of dose effect and kinetics study, and the effects were compared to those of the intraperitoneal (i.p.) route.

Materials and Methods

Animals Specific-pathogen-free male mice of CDF_1 (BALB/c × DBA/2) and C3H/HeJ mice were purchased from Japan SLC, Inc. (Shizuoka). CDF_1 mice were used at 6—8 weeks of age and C3H/HeJ mice were used at 4 weeks of age. These animals were bred under specific-pathogen-free conditions.

SSG The preparation method of SSG has been previously described.[12] SSG contains less than 1% protein and >98% carbohydrate. Lipopolysaccharide contamination of this preparation was less than 0.00014% (1.4 pg/μg of test sample; determined by a chromogenic endotoxin specific assay, ENDOSPECY™, Seikagaku Kogyo, Co., Tokyo).

OK-432 OK-432 (Picibanil, a group of streptococcal preparation) was kindly provided from Chugai Pharmaceutical Co. (Tokyo).

PMs Peritoneal exude cells (PECs) were collected from the peritoneal cavity of CDF_1 mice by washing twice with 5 ml of Hank's balanced salt solution (HBSS; Nissui Seiyaku Co., Tokyo) containing heparin (5 U/ml). After centrifugation, PECs were resuspended in an ice-cold RPMI-1640 medium (Nissui) supplemented with *N*-hydroxyethylpiperazine-*N'*-2-ethansulfonate (HEPES, 5 mM), penicillin G (100 U/ml) and streptomycin (100 μg/ml). The total cell number was counted with a hemocytometer, and cells were differentiated by a Diff-Quik Stain kit (Kokusai Shiyaku Co., Hyogo). Cell numbers of PECs were adjusted to 1×10^6 PMs/ml by an RPMI-1640 medium, incubated for 2 h at 37 °C in a CO_2 incubator and were washed twice with warmed RPMI-1640 medium to remove any nonadherent cells. The resulting monolayers contained the same amount of protein, 20.9 ± 0.2 μg/1×10^5 cells, measured by BCA protein assay reagent (Pierse Co., Rockford, IL), and were used as PMs.

Administration of Samples SSG was dissolved in physiological saline, and aliquots (0.2 ml/mouse) of the solution were administered i.v. (*via* tail vein) or i.p. to CDF_1 mice. In some experiments, the suspension of OK-432 (1 KE) was administered i.p. to CDF_1 mice and PMs were collected 4 d after the injection as a positive control of PM activation.[24]

Assay for Cellular Lysosomal Enzyme Activity Lysosomal enzyme activity was assayed by a method using flat-bottomed 96-well tissue culture plates. PM monolayers in microplates (1×10^5/well) were solubilized by

the addition of 10 μl of 0.1% Triton X-100, then 20 μl of 0.2 M acetate buffer (pH 5.0) were added. Twenty microliters of 24 mM *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) or *p*-nitrophenyl-β-D-glucuronide (Sigma) solution was added as a substrate for acid phosphatase (AP) or β-D-glucuronidase, respectively. After incubation for 30 min at 37 °C, 200 μl of 0.2 M Na_2CO_3 solution (pH 9.8) was added to the reaction mixture, and optical density at 405 nm was measured with a microplate reader (MTP-32, Corona Electric Co., Tokyo).

Assay for Phagocytic Activity Phagocytic activity was assayed by a method described previously.[25] Briefly, PM monolayers (1×10^5/well) were incubated with 5×10^6 of fluorescein-conjugated zymosan particles (prepared from Zymosan A; Sigma) or fluorescein-conjugated zymosan particles opsonized with complement (guinea pig serum), in 96-well flat-bottomed tissue culture plates for 30 min at 37 °C in a CO_2 incubator. PMs were washed twice with warmed RPMI-1640 medium to remove any nonphagocytized zymosan particles, and solubilized by the addition of 100 μl of 50 mM sodium cholate. The fluorescence intensity was measured with a filter fluorometer (microplate reader MTP-32) with an excitation wavelength of 490 nm and emission wavelength of 530 nm. The phagocytic activity was calculated by the following formula; phagocytic acitivity (%) = fluorescein intensity of conjugated fluorescein zymosan/fluorescein intensity of total fluorescein zymosan × 100.

Measurement of H_2O_2 Production PM monolayers in 96-well tissue culture plates (2×10^5/well) were overlaid in 100 μl of Krebs-Ringer phosphate buffer (pH 7.4) containing 30 μM scopoletin, 1 mM NaN_3, 1 purpurogallin U/ml horseradish peroxidase and 100 ng/ml phorbol myristase acetate (Sigma). After incubation for 2 h at 37 °C in a CO_2 incubator, the tissue culture plate was placed in a fluorescence spectrophotometer (microplate reader MTP-32) and fluorescence was recorded with an excitation wavelength of 365 nm and an emission wavelength of 450 nm to measure the H_2O_2 release into buffer.

Measurement of IL-1 Production PM monolayers in 24-well tissue culture plates (2×10^6/well) were incubated for 48 h at 37 °C under 5% CO_2 in 1 ml of an RPMI-1640 medium containing 10% heat inactivated fetal calf serum (Boehriger Mannheim). The supernatant of the cultures was collected, pooled and filtrated though 0.20 μm Millipore filters. IL-1 was quantitated by a double-sandwich enzyme-linked immunosorbent assay (ELISA) as described previously.[26] Briefly, a 96-well plate was coated with hamster anti-rMu IL-1α MAb (Genzyme Co., Boston, MA) in a bicarbonate buffer (pH 9.6). Uncoupled binding sites in the wells were blocked with phosphate buffered saline containing 0.25% bovine serum albumin (Biocell Laboratories Co., Carson, CA) and 0.05% Tween 20 (Wako Pure Chemical Co., Osaka) (BPBST). Wells were incubated with 50 μl of a sample in duplicate for 40 min at 37 °C and then exposed to rabbit polyclonal anti-rMu IL-1α serum (Genzyme). The plate was developed by using peroxidase-labeled goat anti rabbit immunoglobulin G, Fc fragment (Organon Teknika, Co., West Chester, PA) and peroxidase substrate (TMB microwell peroxidase substrate system; Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). Aliquots of rMuIL-1α (Genzyme) dissolved in 50 μl of BPBST were used to construct a standard curve.

Statistics All results an expressed as the arithmetic mean ± standard deviation (S.D.). Statistical evaluations were performed by the Student's *t*-test. A value of $p < 0.05$ was considered significant.

Results

Dose Effect of SSG on Functions of PMs In a previous study, we have shown that SSG administered i.v. had an optimal dose, 250 μg/mouse, for exhibiting antitumor activities in solid form tumor systems.[10,11] Figure 1 shows the dose effect of SSG on functions of PMs. Various doses of SSG (50, 100, 250 or 500 μg/mouse) were administered i.v. on day 0, and activities of PMs were assayed on day 1.[27] As shown in Fig. 1A, all dosages of SSG significantly augmented H_2O_2 production of PMs. The enhancement appeard to be dose dependent in the range from 50 to 250 μg, and dropped at 500 μg. The level of PM activities observed on SSG treated mice were lower than that of OK-432 treated mice (i.p. route), which were the positive control for PM activation. A similar result was observed in the case of lysosomal AP activity, and significant enhancement appeared only at a dose of 250 μg (Fig. 1B). Based on these results, 250 μg/mouse of SSG was administered intravenously in the following experiments.

Changes in the Numbers and Populations of PECs by Administration of SSG Figure 2 shows the numbers and populations of PECs by i.v. or i.p. administration of SSG (250 μg/mouse) during the 12d after administration. In the case of i.v. administration, the numbers of PECs increased from day 2, reached a sharp peak on day 4, and then dropped to the control level on day 8. PM numbers were also increased and changed similarly to that of whole PECs (Fig. 2A). However, the populations of PECs were not changed significantly (Fig. 2B). As compared with this, i.p. administration of SSG increased the numbers of PECs from day 4, and the increased numbers were maintained until day 12. A similar result was observed in the change of PM numbers (Fig. 2C). Before day 2, the numbers of whole PECs were increased temporarily because of the induction of polymorphonuclear leukocytes (PMNs). The percentage of PMNs to whole PECs on day 1 was more than 30% (data not shown) and remained high until day 6 (Fig. 2D). However, the percentage of PMs were not significantly changed by i.p. injection of SSG (Fig. 2D).

Changes of AP Activity of PMs by Administration of SSG Figure 3 demonstrates a kinetics study of the lysosomal AP activity of PMs. SSG (250 μg/mouse) was administered i.v. or i.p. to CDF_1 mice on day 0, and changes in activity were measured until day 12. As a result, i.v. administration of SSG augmented AP activity at two phases (Fig. 3A). In the early phase, enhanced AP activity was observed from day 1, and a peak appeared on day 2. Then, the activity dropped to the control level on day 4, and the second augmentation appeared from day 8. The highest enhancement was observed on day 10, and

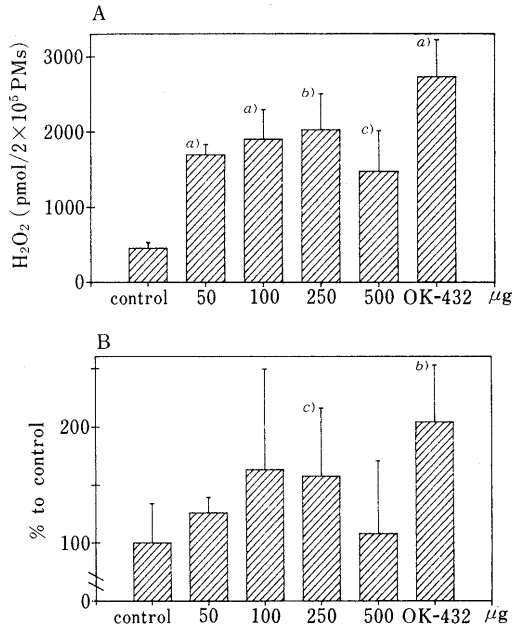

Fig. 1. Dose Effect of SSG on Functions of PMs

Each dose of SSG (50, 100, 250 or 500 μg/mouse) was administered i.v. *via* the tail vein of CDF_1 mice on day 0. OK-432 (1 KE/mouse) was administered i.p. on day 0 as a positive control. PMs were collected on day 1, adhered onto a 96 well microplate (1×10^5 PMs/well) and the activities were assessed. A, H_2O_2 production; B, lysosomal AP activity. Results are expressed as arithmetic mean + S.D. of six mice from two separate experiments. *a*) $p < 0.001$. *b*) $p < 0.01$. *c*) $p < 0.05$.

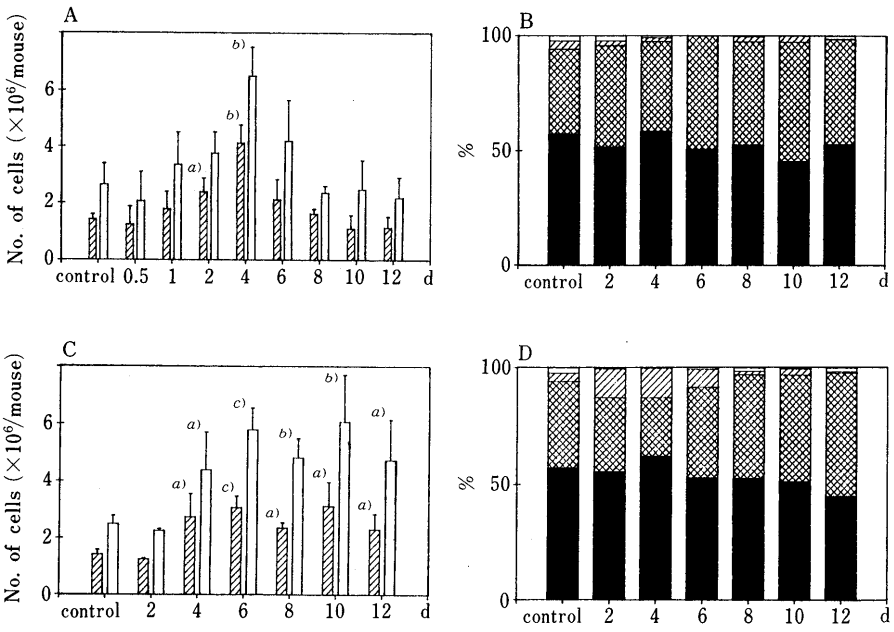

Fig. 2. Changes in the Numbers and Populations of PECs by Administration of SSG

SSG (250 μg/mouse) was administered i.v. (A and B; *via* tail vein) or i.p. (C and D) to CDF_1 mice on day 0. Changes in the numbers (A and C) and populations of PECs (B and D) were measured until day 12. A and C, results are expressed as arithmetic mean + S.D. of three mice; ▨, PM number; □, PEC number. *a*) $p<0.01$. *b*) $p<0.001$. *c*) $p<0.05$. B and D, cell populations are expressed as a percentage of whole PECs; □, eosinophil; ▨, PMN; ⊠, lymphocyte; ■, macrophage; and results are expressed as arithmetic mean of six mice from two separate experiments.

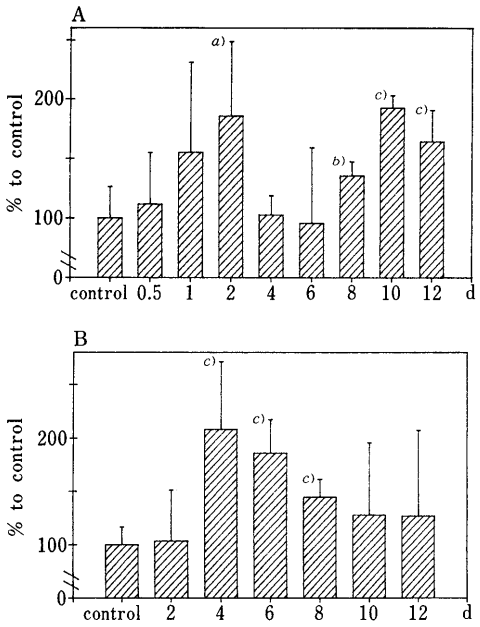

Fig. 3. Changes of AP Activity of PMs by Administration of SSG

SSG (250 μg/mouse) was administered i.v. (A; *via* tail vein) or i.p. (B) to CDF_1 mice on day 0. PMs were collected on each day, adhered onto a 96-well microplate (1×10^5 PMs/well) and changes of lysosomal AP activity were observed until day 12. Results are expressed as arithmetic mean + S.D. of five mice. *a*) $p<0.05$. *b*) $p<0.01$. *c*) $p<0.001$.

decreased gradually on day 12. In contrast, SSG administered i.p. augmented the activity at only one phase, day 4, and a broad peak appeared until day 8 (Fig. 3B).

Effect of i.v. Administration of SSG on Various Functions of PMs It has often been reported that augmented functions, such as phagocytosis, lysosomal enzymes, oxidative responses and monokine production, were observed on activated PMs, and these macrophage functions play important roles on various immune responses.[18,28,29] We examined the effect of i.v. administration of SSG on these functions. SSG (250 μg/mouse) was administered i.v. on day 0, and the functions were assayed on days 2 and 10, which showed peaks in activity demonstrated by the AP activity in Fig. 3. As shown in Table I, phagocytic activity of PMs was enhanced on both days 2 and 10 when either opsonized or unopsonized zymosan particles were used. A similar result was observed in lysosomal AP activity and β-D-glucuronidase activity. An oxidative response, H_2O_2 production, of PMs was significantly augmented by SSG only on day 2, and the activity was reduced to the normal level on day 10. SSG was also enhanced a monokine, IL-1α, production of PMs, but only on the second day after the administration. However, OK-432, a positive control for PM activation, did not affect IL-1α production of PMs.

Discussion

Macrophages play a major role in immune responses and are important effector cells for immunomodulating effects of (1→3)-β-D-glucans.[4,10-13,15,20] In this paper, we characterized the activities of macrophages obtained from the peritoneal cavity after i.v. or i.p. administration of SSG (250 μg/mouse) to CDF_1 mice, and concluded that the characteristics of macrophage activation were significantly dependent on administration routes of SSG.

In the case of i.p. administration, SSG increased and activated PMs from day 4, and this activity was maintained until days 8—12 (Figs. 2C, D and 3B). In contrast, SSG administered i.v. increased the numbers of PMs at one phase, a sharp peak appeared on day 4 (Fig. 2A) without changing the populations of PECs (Fig. 2B), and PMs were activated at two phases, on days 2 and 10 (Fig. 3A). These differences would be due to the different distribution of SSG depending on administration routes. In the case of i.p. injection, the majority of SSG remained in the peritoneal cavity for more than a day (manuscript in

TABLE I. Effect of SSG on Various Functions of PM[a]

Sample[b]	Day[c]	Phagocytic activity		Lysosomal enzyme activity		H_2O_2 production (pmol)[e]	IL-1α production[f] (pg)[g]
		Unopsonized (%)	Opsonized (%)	AP (PNP μmol)[d]	β-D-Glucuronidase (PNP nmol)[d]		
Control	—	6.8±0.5	12.8± 1.6	0.9±0.2	233.2± 65.0	385.5±129.2	9.7
SSG	2	11.2±0.8[h]	20.4± 8.2[j]	1.8±0.6[j]	319.5± 36.2[j]	1477 ±700.4[i]	12.1
SSG	10	11.9±1.2[i]	21.6± 6.2[h]	1.9±0.2[i]	362.0± 25.7[h]	778.3±816.5	8.1
OK-432	4	22.9±4.1[j]	39.8±20.0[h]	5.7±0.6[i]	812.2±215.9[j]	2333 ±624.0[i]	8.3

a) Results are expressed as arithmetic mean±S.D. of seven mice from two separate experiments. *b*) SSG (250 μg/mouse) was administered i.v. on day 0. OK-432 (1 Klinische Einheit/mouse) was administered i.p. on day 0 as a positive control for PM activation. *c*) Days after the administration of samples. *d*) μ or nmol of *p*-nitrophenol/30 min per 1×10^5 PMs. *e*) pmol of H_2O_2/2 h per 2×10^5 PMs. *f*) Results are expressed as arithmetic mean of duplicate dishes. *g*) pg/1×10^6 PMs. *h*) $p<0.01$. *i*) $p<0.001$. *j*) $p<0.05$.

preparation) and caused inflammatory-like responses because inflammatory leukocytes, PMNs, were exuded into the peritoneal cavity in an early phase after the injection of SSG (data not shown). This PMN accumulation was restored gradually after day 2, and the PM numbers were increased from day 4 (Figs. 2C, D). These cellular changes were typical of an inflammatory-like response caused in the peritoneal cavity, and it was reported that PMs were activated during these responses.[24] Therefore, the augmented PM activity by i.p. administration of SSG might be mediated by these inflammatory-like responses. Additionally, it has been reported that soluble (1→3)-β-D-glucan activated the functions of PMs, such as lysosomal enzymes, *in vitro*,[28] and SSG could also stimulate PMs *in vitro*.[30] These findings also suggested that SSG administered i.p. stayed in the peritoneal cavity for a long period and could stimulate PMs directly; therefore, SSG could enhance the PM functions both quantitatively and qualitatively for a duration of several days. In contrast, SSG administered i.v. might not be able to stimulate PMs directly, because SSG would be distributed immediately to some systemic reticuloendothelial tissues, such as the liver and spleen.

It could not be clearly explained why i.v. administration of SSG activated PMs at 2 phases, but the following speculations could be possible. The first peak of PM activation which appeared on day 2 might be, at least in part, due to the acute phase responses. We have previously reported that SSG induced some acute phase responses, including activation of complement[30] and hematopoietic responses.[14] These responses are induced during a couple of days after the administration of SSG, and it was also well known that macrophages are activated within these acute phase responses.[31,32] The second peak of PM activation which appeared on day 10 may be dependent on augmentation of systemic immune responses, including lymphocyte activation. It was reported that about 10 d are needed to induce the systemic immune responses mediated by the cytokine network after the stimulation of BRMs,[23] and macrophages were activated by various cytokines released from lymphocytes and/or from the macrophages themselves during these systemic immune responses.

Further examinations to demonstrate the relationship between administration routes of BRMs and activation of macrophages obtained from different tissues would be of great value since it has been reported that macrophage activation *in vivo* by BRMs differed depending on administration routes of BRMs and on populations of macrophages.[21,22,24,33,34] We reported previously that a single i.p. injection of SSG (250 μg/mouse) augmented the functions of alveolar macrophages (AMs) at two phases, on days 1 and 8.[15] SSG also enhanced the functions of both PMs[13] and AMs[35] when administered orally for 10 consecutive days at a dose of 2000 μg/mouse. Similar studies have been reported using other BRMs such as OK-432,[24,33] BCG,[21] muramyl dipeptide[22] and kampomedicines,[34] and these data might be important to clearly understand the mechanisms of macrophage activation *in vivo* by BRMs.

As shown in Table I, SSG administered i.v. enhanced lysosomal enzyme activity, phagocytic activity and H_2O_2 production of PMs. These scavenger functions of PMs are responsible for killing micro-organisms and cancer cells *in vivo*. The antitumor activity of i.v. administration of SSG may be partly due to these scavenger functions of macrophages. In this study, single i.v. administration of SSG activated PMs at a dose of 250 μg/mouse (Fig. 1) also exhibited antitumor activities in solid form tumor systems.[10,11] Multiple administrations were required to exhibit optimum antitumor activity. These findings suggested that macrophages would be stimulated to a more active stage by multiple administration of SSG.

The increased IL-1α secretion from PMs was observed only on the second day after the i.v. administration of SSG (Table I), though no significant enhancement of IL-1α production was detected from PM lysates on any day (data not shown). These data suggest that IL-1α was secreted from PMs into the culture medium in an early phase after the i.v. administration of SSG. IL-1 plays a key role in the cytokine network and is important to the T-cell activation in systemic immune responses[23,26]; therefore, increased IL-1 release from PMs by i.v. administration of SSG will be able to induce more cellular immune responses. The augmentation of antitumor activities by SSG may be due to IL-1, and the PM activation which appeared in an second peak, on the 10th day after i.v. administration of SSG, also may be partly due to IL-1α released from PMs on the second day.

The augmented H_2O_2 production from PMs was also observed only on the second day, though it was not observed on the 10th day after the i.v. administration of SSG (Table I). In contrast, the change of PM numbers by SSG was different from the kinetics of PM activities (Figs. 2A and 3A). Considering these findings, the characteristics

of PM activation by SSG was suggested to be dependent on various kinds of activities, and it supported the presence of some different mechanisms to activate PMs by i.v. administration of SSG *in vivo*.

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