Modulation of the Antitumor Effect and Tissue Distribution of Highly Branched (1→3)- β -D-Glucan, SSG, by Carrageenan

Masahiro SUDA, Naohito OHNO, Yoshiyuki ADACHI, and Toshiro YADOMAE*

Laboratory of Immunopharmacology of Microbial Products, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan. Received October 27, 1994; accepted January 27, 1995

The action of carrageenan (CAR), a representative blocking reagent for phagocytes, on the antitumor effect and tissue distribution of highly branched (1→3)- β -D-glucan, SSG, was examined. CAR inhibited the antitumor effect of intraperitoneally administered SSG only when applied before inoculation of the tumor, and had little effect when applied after tumor inoculation. A similar result was observed when SSG was administered intrasplenically. In contrast, CAR had considerable effect on tissue distribution of i.p. SSG. The differences with respect to the results in normal mice were: 1) the distribution of SSG from the peritoneal cavity to the rest of the body was inhibited, 2) large numbers of peritoneal exudate cells (PEC) were produced and a relatively high concentration of ^3H -SSG was found in the PEC fraction 48 h after administration of ^3H -SSG, 3) one week after administration, ^3H -SSG was distributed to throughout the body but the amount of ^3H -SSG distributed was lower than in normal mice, 4) a significant amount of ^3H -SSG was recovered from ligaments (containing omental milky spots, peritoneum, mesentery and associated fat) in which negligible amounts were found in normal mice. These results suggest that the inhibition of the antitumor effect of SSG by CAR probably results from the prevention of the natural resistance of mice which is related to phagocytic function, and that the distribution of SSG to throughout the body is significantly modulated by CAR.

Key words antitumor glucan; *Sclerotinia sclerotiorum*; ^3H -labeled (1→3)- β -D-glucan (^3H -SSG); carrageenan; antitumor effect

(1→3)- β -D-Glucans, such as lentinan (LTN)¹⁾ and schizophyllan (SPG)²⁾ have been used clinically in cancer therapy in Japan. It is well-known that the antitumor effect of these glucans is mediated by augmentation of the host immune system.³⁾ In fact, it is reported that (1→3)- β -D-glucans activate macrophage function,^{4–6)} adjuvant activity,⁴⁾ blood coagulation system,⁷⁾ hematopoietic response,⁸⁾ complement system,^{4,9)} and NK cell activity.¹⁰⁾

Recently, several investigators have suggested that there are β -glucan binding proteins on human monocytes and murine peritoneal macrophages (PM) and these proteins may be candidates for the functional β -glucan receptor.^{11,12)} It is also reported that the complement receptor type 3 (CR3) recognizes (1→3)- β -D-glucans.¹³⁾ These reports suggest that monocytes/macrophages have an important action on the antitumor effect of (1→3)- β -D-glucans. In addition, these cells are thought to participate in the tissue distribution of β -glucans.¹⁴⁾

We have prepared metabolically ^3H -labeled SSG (^3H -SSG),¹⁵⁾ obtained from the liquid-cultured broth of a fungus *Sclerotinia sclerotiorum* IFO 9395, and studied the relationships between the antitumor and immunomodulating effects and pharmacokinetics of SSG. In a previous paper,¹⁶⁾ we reported that about half the ^3H -SSG administered intraperitoneally (i.p.) was moved into liver and spleen, and almost all of the distributed SSG remained in the body for at least one month during which time most of the immunomodulating effects of SSG were significantly reduced. In addition, it was unexpected that each cell fraction, which included Kupffer cells and splenic macrophages, contained only about 10% of the distributed SSG in each tissue. These results seem to suggest that the monocyte/macrophage lineage cells may be less important for the distribution of (1→3)- β -D-glucans. However, considering the hypothesis of the β -glucan receptor and

the possibility of oxidative degradation of (1→3)- β -D-glucans,¹⁷⁾ the role of the monocyte/macrophage lineage can never be ignored.

Previously, we reported that treatment of mice with carrageenan (CAR), a representative blocking agent for the phagocytic system, before tumor inoculation abrogated the antitumor effect of a (1→3)- β -D-glucan, GRN, obtained from *Grifola frondosa*.¹⁸⁾ In this study, we examined the effect of CAR, with particular attention to the role of the monocyte/macrophage lineage, on the antitumor effect and tissue distribution of SSG.

MATERIALS AND METHODS

Materials CAR (Type II, C-1138) was purchased from Sigma Chem. Co., St. Louis, MO.

Mice Male ICR mice were obtained from Japan SLC, Inc., Shizuoka. These mice were 5–10 weeks of age and placed under specific pathogen-free conditions.

Tumor Sarcoma 180 tumor cells were maintained in the peritoneal cavities of ICR mice.

Evaluation of Antitumor Effects of SSG ICR mice received s.c. inoculations of sarcoma 180 tumor cells (5×10^6) into the right hind groin on day 0. The indicated doses of SSG were administered i.p. or intrasplenically (i.s.) on days 1, 3, 5, 7 and 9. Control mice were given physiological saline instead of the test solution. On day 35, the mice were sacrificed and each tumor was weighed. The significance (p value) of any differences between the control and treated groups was evaluated using Student's t -test.

Assay for Distribution of ^3H -SSG in Various Tissues After the administration of ^3H -SSG, the mice were sacrificed by exposure to ether and mouse blood was collected by cardiac puncture. Then the peritoneal cavity

* To whom correspondence should be addressed.

was washed by Hank's solution and several tissues were removed. Liquid such as urine was collected by absorption onto the filter paper. Each tissue was dried in air overnight and the dried tissues were oxidized by combustion in an automatic sample oxidizer (ASC-113, Aloka). The $^3\text{H}_2\text{O}$ formed after combustion was measured in a liquid scintillation counter.

RESULTS

Effect of CAR on the Antitumor Effect of SSG To confirm that the antitumor effect of SSG was abrogated by CAR treatment, CAR (4 mg/0.8 ml/mouse) was administered i.p. before (days -5, -3 and -1) or after (days 1, 3 and 5) the tumor inoculation, and SSG (25, 100 or 250 μg) was administered i.p. or i.l. on days 1, 3, 5, 7 and 9. As shown in Table 1, when CAR was administered before tumor inoculation, the antitumor effect of SSG was significantly abrogated. However, when CAR was administered after tumor inoculation, the inhibition of the antitumor effect of SSG was reduced. This result is consistent with that obtained with GRN.¹⁸⁾ Furthermore, the fact that the antitumor effect of i.l. SSG was also abrogated by CAR treatment supports the importance of the phagocytic system especially in the early stage of this model. Similarly, the above concept is supported by the fact that CAR treatment after tumor inoculation, that is after recognition and presentation of tumor antigen by macrophages, had little effect on the antitumor effect of SSG.

Effect of CAR on the Tissue Distribution of SSG CAR is known not only as a macrophage blocking agent but also as a potent inflammatory agent. In fact, i.p. CAR induces a considerable number of peritoneal exudate cells (PECs). Since monocyte/macrophage lineage is thought to play an important role on tissue distribution of (1 $\rightarrow$ 3)- β -D-glucan,¹⁴⁾ it is interesting to investigate whether CAR changes the tissue distribution of SSG. As shown in Table 2, large amounts of i.p. ^3H -SSG remained in the peritoneal cavity 48 h after administration. Comparing our previous data showing that the half clearance time of ^3H -SSG from the peritoneal cavity was about 8 h and only 10% remained at 48 h in normal mice,¹⁶⁾ CAR treatment significantly inhibited the distribution of ^3H -SSG from the peritoneal cavity to rest of the body. Furthermore, significant amounts of ^3H -SSG were detected in the PEC fraction (at 48 h, ten times higher than in normal mice). The number of induced PEC was also ten times higher (5.0×10^6 cells/mouse in normal mice, 5.3×10^7 cells/mouse in CAR-pretreated mice), and the capacity of each PEC to incorporate ^3H -SSG (μg SSG/ 10^6 cells) was similar to normal mice (about 0.7 $\mu\text{g}/10^6$ cells and 0.5 $\mu\text{g}/10^6$ cells, respectively). On the other hand, the distribution to liver, spleen and kidney, which were the main tissues for ^3H -SSG distribution in normal mice, was quite low.

At 7 d after administration of ^3H -SSG, significant amounts of ^3H -SSG had migrated from the Sup and PEC fractions to ligaments. The ligaments in this case included mesentery, peritoneum and associated fat. In ligaments, there were omental milky spots and one of the lymphoid systems consisting of macrophages and lymphocytes

Table 1. Effect of CAR on the Antitumor Activity of SSG

Group	CAR (4 mg/0.8 ml)	SSG ($\mu\text{g} \times 5$)	Tumor weight (g, mean $\pm$ S.D.)	Inhibition ratio (%)	Complete regression
1	Nil	0 (i.p.)	2.75 ± 1.72	—	0/10
2		25	0.30 ± 0.55^a	89	6/11
3		100	0.32 ± 0.80^b	88	6/10
4		200	0.13 ± 0.19^c	95	6/11
5	-5, -3, -1	0 (i.p.)	10.56 ± 5.20	-284	0/8
6		25	5.63 ± 5.35	-104	0/6
7		100	5.41 ± 6.00	-97	0/8
8		250	2.05 ± 3.37^d	25	2/7
9	-5, -3, -1	0 (i.l.)	8.66 ± 7.80	-215	0/8
10		25	4.59 ± 5.49	-76	2/9
11		100	2.44 ± 5.28	11	2/6
12		250	2.27 ± 5.31	17	1/8
13	1, 3, 5	0 (i.p.)	4.60 ± 2.92	-67	0/9
14		25	2.32 ± 1.34	16	0/5
15		100	$0.95 \pm 0.90^{a,d}$	65	1/4

Sarcoma 180 (5×10^6 cells) was inoculated into the right groin of ICR mice on day 0. SSG was administered on days 1, 3, 5, 7 and 9. On day 35, tumor masses were weighed. Significant differences from control (group 1) were evaluated using Student's *t*-test; a) $p < 0.05$, b) $p < 0.01$, c) $p < 0.001$. Significantly different from the corresponding control group (group 5, 9, or 13); d) $p < 0.01$.

Table 2. Effect of CAR on the Tissue Distribution of SSG

	4 h	8 h	24 h (% of total)	48 h	7 d
Blood	1.72	0.16	0.38	0.66	0.30
Liver	0.27	1.44	0.81	0.60	4.83
Kidney	0.07	0.09	0.10	0.18	0.73
Heart	0.00	0.00	0.02	0.03	0.04
Spleen	0.04	0.04	0.10	0.22	0.76
Stomach	0.16	0.12	0.12	0.59	0.46
Intestine	0.54	0.89	0.65	0.90	5.40
Lung	0.03	0.03	0.02	0.03	0.13
Ligament	0.80	1.71	1.75	0.21	29.77
Sup	64.37	61.47	57.21	36.23	1.12
PEC	3.06	3.18	5.77	8.20	1.56
Feces	0.00	0.03	0.05	0.33	N.D.
Excrement	0.13	0.16	0.39	0.49	N.D.

Carrageenan (4 mg/0.8 ml) was administered i.p. on day -2. Two hundred and 50 μg ^3H -SSG [about 100000 dpm (total ^3H -SSG)] was administered i.p. on day 0. Each tissue was removed at the indicated time and oxidized. The data are shown as averages of three experiments and expressed as follows; percentage of total = {dpm[tissue]/dpm[total ^3H -SSG]} $\times$ 100. Sup is the supernatant fraction after centrifugation of the peritoneal exudate cell suspension recovered by washing the mouse peritoneal cavity with Hank's solution. PEC is the peritoneal exudate cell pellet obtained after centrifugation. Ligament includes omentary milky spot, mesentery, peritoneum and associated fat. Excrement is the liquid excrement collected by absorption into the filter paper. N.D.; not done.

aggregated around blood capillaries. Interestingly, the distribution of SSG to ligaments was quite low in normal mice (less than 1% of administered SSG, manuscript in preparation).

When CAR-induced PECs were labeled with ^{51}Cr *ex vivo* and inoculated i.p. into other CAR-treated mice, then 24 h later, most of the ^{51}Cr -labeled PECs remained in the peritoneal cavity and the rest had migrated to ligaments (about 8%, data not shown). Therefore, the accumulation of ^3H -SSG in ligaments would be the result of the aggregation of PECs incorporating ^3H -SSG in the ligaments.

Tissue Distribution of ^3H -SSG in Tumor-Bearing Carrageenan-Treated Mice The effect of CAR treatment

Fig. 1. Effect of the Timing of CAR Treatment on the Tissue Distribution of ^3H -SSG

Sarcoma 180 (5×10^6 cells) was inoculated into the right groin of ICR mice on day 0. Two hundred and $50 \mu\text{g}$ ^3H -SSG (about 100000 dpm) was administered i.p. to the mice on days 1, 3 and 5. One (▨), 2 (■), 3 (□) or 4 (▤) weeks after tumor inoculation, each tissue was removed and oxidized in an automatic sample oxidizer (ASC-113, Aloka). The data are shown as the average of four experiments and expressed as follows; percentage of total = $\{\text{dpm}[\text{tissue}]/\text{dpm}[^3\text{H}\text{-SSG}]\} \times 100$. Sup is the supernatant fraction after centrifugation of the peritoneal exudate cell suspension recovered by washing the peritoneal cavity with Hank's solution. PEC is the peritoneal exudate cell pellet obtained after centrifugation. Ligament included mesentery, peritoneum and associated fat. (A) CAR (4 mg/0.8 ml/mouse) was administered i.p. before tumor inoculation (days -5, -3 and -1, in this system, the antitumor effect of SSG was significantly abrogated). (B) CAR was administered i.p. after tumor inoculation (days 1, 3 and 5, in this system, the antitumor effect of SSG was observed).

on the tissue distribution of ^3H -SSG was examined using tumor-bearing mice. The same experimental system shown in Table 1 was used in this experiment. Figures 1A and 1B show the tissue distribution of ^3H -SSG in mice treated with CAR before (Fig. 1A) or after (Fig. 1B) tumor inoculation, respectively. Comparing both systems, the distribution of ^3H -SSG to liver in Fig. 1B was only slightly higher than that in Fig. 1A. Since much of ^3H -SSG was distributed to ligaments in both systems, the distribution of SSG to ligaments would be irrelevant to the abrogation of the antitumor effect of SSG by CAR.

DISCUSSION

In this paper, we examined the action of carrageenan on the antitumor effect and tissue distribution of SSG. We demonstrated that 1) the antitumor effect of SSG, administered not only intraperitoneally but intralesionally, was abrogated by CAR treatment just before tumor inoculation, 2) the distribution of ^3H -SSG to the entire body was prevented by CAR treatment, 3) the distribution of ^3H -SSG to the PEC fraction in CAR-treated mice was higher than that in normal mice, 4) significant amounts of ^3H -SSG distributed to ligaments were not related to the antitumor effect.

The effect of CAR abrogating the antitumor effects of BRMs (biological response modifier) has been reported in the case of *Lactobacillus* LC-9018,¹⁹⁾ *Bacillus* of Calmette and Guerin (BCG) and *Corynebacterium parvum*.²⁰⁾ Since macrophages are thought to play key roles in most BRM activities, these effects of CAR are not surprising. However, it is very important to clarify the mode of action of CAR more precisely. The results shown in Table 1 suggest that, especially in the very early stage of this

system, when macrophages would recognize and present tumor antigen, abrogation by CAR took place. In addition, the natural resistance of the host to the tumor was damaged by this treatment and thus the tumor weights of the treated groups (groups 5, 9 and 13) were significantly increased compared with group 1. CAR treatment increased the number of PEC more than ten-fold and thus this would result in a relative decrease in immune effector cells, including macrophages, infiltrated into the tumor.

Based on this basic mechanism, the loss of the antitumor activity of SSG (both by systemic and local administration) following CAR treatment was significantly related to the reduced natural resistance of the host to the tumor produced by CAR treatment. Interestingly, even in this reduced host defense system, a certain degree of inhibition of tumor growth was observed following by SSG administration (groups 8 and 15). This effect could be very important for the clinical application of BRMs in immune compromised patients.

The most exciting observation reported in this paper is that the distribution of systemically administered ^3H -SSG significantly depends on whether CAR treatment has been given or not. Most of the administered SSG remained in the peritoneal cavity, including peritoneal exudate cell, omental milky spot, fat and other ligaments in CAR-treated mice. Several experiments suggested that this result from aggregation of the ^3H -SSG-incorporated PEC. However, since the distribution to ligaments was observed in both ineffective (Fig. 1A) and effective (Fig. 1B) systems, this distribution cannot be closely related to the activities of SSG.

Recently, more selective methods to block monocyte/macrophage lineage, such as gadolinium chloride (GdCl_3)^{21,22)} and dichloromethylene diphosphonate en-

capusurated liposome (Cl₂MDP-liposome),^{23,24)} have been reported. It would be very interesting to compare the effects of these methods and CAR on the antitumor effect and tissue distribution of (1→3)-β-D-glucans. Such comparison may clarify the critical roles of monocyte/macrophage lineage in various aspects of the action of (1→3)-β-D-glucans.

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