Inactivation of a Particle β -Glucan by Proteins in Plasma and Serum

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(1→3)- β -D-Glucans remained in the liver and spleen for long time, *i.e.* more than a month, without major structural changes/because there is no specific metabolic pathway for it in the body. However, biological activities, such as priming activity to LPS, triggered TNF- α synthesis, and antitumor activity was reduced more quickly. In this paper, we demonstrated the contribution of protein binding in inactivating β -glucans. A particle β -glucan preparation, zymosan, was treated with serum or plasma at 37 °C and their various biological activities were compared with zymosan alone. Such biological activities as antitumor activity, TNF-production, IL-6 production, complement activation and vascular permeability were significantly decreased by serum or plasma treatment. These results strongly suggested that the binding of serum or plasma protein(s) to β -glucans would be a key step in inactivating a particle β -glucan in the body.

Key words (1→3)- β -D-glucan; inactivation; protein binding

(1→6) Branched (1→3)- β -D-glucans (β -glucans) show various activities, such as antitumor activity, and some of them have been clinically applied to cancer patients in Japan. Many of the biological activities, including antitumor activity,¹⁾ haematopoietic activity,²⁾ and antimetastatic activity,³⁾ were found, in detailed examinations, to be induced by the activation of the self defense system. β -Glucan receptors have been suggested to be present on human monocytes and murine macrophages, and these are thought to be candidates as functionally important receptors.⁴⁾

In a series of experiments, we have demonstrated that two systematically administered β -glucans, SSG (from *Sclerotinia sclerotiorum* IFO 9395) and GRN (from *Grifola frondosa*), were retained in the body, especially in the liver and spleen, for a very long period, *i.e.* more than a month,⁵⁾ without significant structural changes. However, we also observed that certain biological activities, such as increased celuroplasmin concentration, and macrophage activation, disappeared within a month following the β -glucan administration.⁶⁾ These observations are consistent with the concept that a certain inactivation mechanism other than chemical/physical degradation of the overall structure exists.

Zymosan (ZYM), originally prepared from a yeast and mainly composed of a particulate (1→3)- β -D-glucan, shows a variety of biological activities, some of them similar to soluble β -glucans, and others not. One of the most significant differences in water soluble and insoluble β -glucans relates to the production of active oxygen species and arachidonic acid from the mononuclear phagocytes. Actually, water soluble β -glucans inhibit the particulate β -glucan mediated synthesis of hydrogen peroxide from murine peritoneal macrophages. In addition, the fact that the administered particulate β -glucan *in vivo* was effectively removed from circulation by the phagocytic cell has been well characterized, but that of soluble β -glucan has not been examined in detail.^{7–11)} To confirm the fate of β -glucans and the duration of their activity in mice, we

examined the antitumor activity of β -glucans by various treatment schedules. We determined the concentration of β -glucans in the animals and found that, similar to the soluble β -glucans, the antitumor activity of β -glucans was reduced at an early stage, though β -glucans of a high concentration remained in the spleen or liver.

From analyses of the deposited β -glucans in ICR mice by chemical, physical, and biochemical methods, it has been found that the molecular weight was gradually reduced and acidic groups were incorporated into the molecules. These facts strongly suggest that the β -glucans are degraded mainly by oxidative degradation mediated by products of phagocytes.¹²⁾ Blocking of the functions of phagocytes by gadolinium chloride reduced the degradation rate.¹³⁾ However, it was also found that β -glucan fractions extracted from the liver are still active, as assessed by antitumor activity.¹⁴⁾ These facts again suggest that the involvement of certain inactivation mechanisms other than chemical or physical degradation takes place in organs.

It is a generally accepted concept of drug metabolism that protein-bound substances, such as by serum albumin, are inactive, especially in low molecular weight medicine. It is probable that a similar mechanism also proceeds in high molecular weight, as well as particulate substances. In this report we compared the immunopharmacological activities of β -glucan, especially a particulate β -glucan, with its protein bound form. Immunopharmacological activities are known to be regulated by various factors, such as both acute and chronic inflammatory responses. Thus, we examined several activities and discussed the contribution of protein binding to the *in vivo* activity.

MATERIALS AND METHODS

Mice Male ICR, 5 weeks old, were obtained from Japan SLC, Inc. (Shizuoka, Japan).

Tumor Sarcoma 180 tumor cells were maintained serially in ascites form in ICR mice.

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Cell Macrophage cell line RAW 264.7 cells (obtained from RIKEN CELL BANK, Tsukuba, Japan) were maintained in a plastic culture flask in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Bioserum, Victoria, Australia) (complete RPMI medium). For experiments, the cells were removed from the culture flask by wiping the bottom of the flask with a CELL SCRAPER (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and were centrifuged (1200 rpm, 5 min). Then the cells were washed with RPMI medium to a concentration of 5×10^5 cells/ml.

Glucans ZYM (Zymosan A) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Zymocel (ZYC) was from Wako Chemical (Tokyo, Japan). SPG (Sonifiran prepared from schizophyllan) was provided from Kaken Pharmaceutical Co., Ltd. (Tokyo). SSG and GRN were prepared as described previously.

Serum and Plasma Mouse blood was obtained by cardiac puncture. After the blood was centrifuged at $2000 \times g$ for 15 min, the supernatant was used as normal mouse serum. The mouse blood was mixed with heparin, centrifuged at $2000 \times g$ for 15 min, and the supernatant was used as normal mouse plasma.

Glucans Treated by Serum or Plasma Serum or plasma and ZYM (10 mg/ml) or ZYC (10 mg/ml) were mixed in a volume ratio of 1:1, and was incubated at 37°C for 30 min. Particles were collected by centrifugation and washed with saline 3 times.

Evaluation of Antitumor Activity Antitumor activity were evaluated against the solid form of Sarcoma 180 tumor cells in ICR mice. Tumor cells (5×10^6) were inoculated subcutaneously into the groin of mice. β -Glucans were administered by intravenous (i.v.) injection or intraperitoneal (i.p.) injection. Antitumor activity was evaluated by weighing the tumor 5 weeks after inoculation. Inhibition (%) was calculated from the mean weight of the control group.

Assay of TNF- α and IL-6 TNF- α and IL-6 levels of RAW 264.7 culture supernatants were measured using an enzyme-linked immunosorbent assay (ELISA). Five $\mu\text{g/ml}$ of anti mouse TNF- α or IL-6 mAb (Pharmingen, San Diego, CA, U.S.A.) was bound to the surface of 96-well, flat bottomed plates (Sumitomo Bakelite Co., Tokyo, Japan) by incubation at 4°C overnight in Ca^{2+} and Mg^{2+} -free phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST) and blocked with 0.5% bovine serum albumin in PBST at 37°C for 40 min. After being washed with PBST, the plate was incubated with rMu TNF- α (rMu IL-6) (R & D Systems, Minneapolis, MN, U.S.A.) or the samples, and then reacted with a 1/2000 dilution of anti-TNF- α or anti-IL-6 rabbit polyclonal antibody (Genzyme, Boston, MA, U.S.A.). After washing and blocking, the plate was treated with a 1/10000 dilution of peroxidase-labeled anti-rabbit IgG (Enzyme, Boston, MA, U.S.A.). After final washing, a peroxidase-conjugated antibody was detected by the addition of a TMB (3,3',5,5'-tetramethylbenzidine) substrate system (KPL, Inc., Gaithersburg, MD, U.S.A.). Color development was stopped with 1 N phosphoric acid and the absorbance at 450 nm was measured with a microplate reader (MTP32, Corona Electric Co., Ltd., Tokyo, Japan).

Complement Activity Blood was obtained from healthy volunteers and was maintained at room temperature for 2 h. The blood was then centrifuged at 3000 rpm for 15 min, and the supernatant was used as the normal human serum (NHS). NHS (25 μl) and VEM (veronal buffered saline (VBS) containing ethylene glycol bis(2-amino ethyl-ether)*N,N,N,N*-tetraacetic acid and MgCl_2 , 5 μl) were mixed in a 96-well plate for ELISA, and the mixture was incubated at 37°C for 10 min. In the resulting mixture, 10 μl of various preparations of ZYM were added, and the mixture was incubated at 37°C for an additional 30 min. After the incubation, 50 μl of rabbit red blood cells (7.5×10^8 cells/ml) was added to this mixture, and the plate was incubated at 25°C . The turbidity of rabbit red blood cell (RRBC) was measured by a microplate reader at 630 nm every 15 min.

Vascular Permeability A stimulant (100 $\mu\text{g}/\text{mouse}$), such as ZYM, ZYM treated with serum or treated by plasma, was i.p. injected into the mice. Thirty min after the injection, an aqueous solution of Evans blue (Sigma E-2129; 10 mg/ml in saline) was injected *via* an i.v. route (0.2 ml). Thirty min later, the mice were sacrificed and the peritoneal exudated fluids were collected by lavaging with 4 ml of PBS. After centrifugation to remove any debris, the concentration of Evans blue in the peritoneal cavity was measured at 630 nm.

RESULTS

Complement Activity Complement is important for both opsonization and the production of anaphylatoxins. Complement activation is strongly related to the following immune and inflammatory cascades. Thus, we examined the complement activation, vascular permeability, and activation of macrophages by serum treated β -glucan.

To compare the complement activation by β -glucan and serum-treated β -glucan, we used ZYM in the presence or absence of serum treatment. Complement activation was

Fig. 1. Effects of Protein Binding on Complement Activity by ZYM

ZYM was treated with normal human serum as follows: No. 1, ZYM (10 μl) treated with VEM; No. 2, ZYM (10 μl) treated with NHS (40 μl); No. 3, ZYM (10 μl) treated with heat inactivated NHS (40 μl); No. 4, ZYM treated with NHS (40 μl) was then heat inactivated at 56°C , 30 min treatment for inactivating complement. Treated sera were mixed with RRBC and incubated at 25°C for 10 min to assess remaining complement activity.

measured by the lysis of RRBC. As shown in Fig. 1, the serum incubated with ZYM (No. 1) showed almost no lysis of RRBC because of the consumption of the complement components during incubation. In contrast, the serum incubated with serum- or plasma-treated ZYM (No. 2) still induced the lysis of RRBC. Loss of the binding capacity by the heat treatment (Nos. 3, 4) correlated well with the property of the complement component. These facts strongly suggest that once treated, ZYM has no activity to produce active complement-fragments.

Vascular Permeability Vascular permeability (VP) is a very sensitive parameter for monitoring acute inflammation. VP is increased by many mechanisms and substances, including complement activation, histamine release, and prostaglandins synthesis. Thus, we compared the VP of the peritoneal cavity after the intraperitoneal administration of ZYM and serum- or plasma-treated ZYM. We measured the VP 1 h after ZYM administration. As shown in Fig. 2, VP was significantly increased by treatment with ZYM, and the activity was reduced by both in the cases of serum treated- and plasma treated-ZYM. These facts strongly suggest that at least half of the VP increasing activity was strongly related to the protein binding.

TNF- α and IL-6 Assay *in Vitro* The cells at the ZYM administration site (peritoneal cavity) are important in assessing the local reaction as well as the systemic reactions. We compared the cytokine synthesis of the peritoneal macrophage by ZYM and serum- or plasma-treated ZYM. Figs. 3A and 3B showed TNF- α and IL-6

syntheses. Cytokine synthesis was also inhibited by protein binding. These facts strongly suggested that the cell mediated activity was also reduced, at least a part, after the protein binding.

Organ Weight Administration of ZYM i.v. induced dilation of the spleen, and the weight of the spleen increased. Thus, spleen and liver weights were compared 1 week after ZYM or serum (plasma)-treated ZYM administration. The dose of ZYM administered was 1 mg/mouse, which was previously determined to be optimum for the antitumor activity. When ZYM was treated with serum or plasma, the spleen was smaller than that with ZYM treatment alone. The liver weight was increased by ZYM administration, but the increase was similar to the cases of protein binding. Reduced splenomegaly might

Fig. 2. Effects of Protein Binding on Production of TNF- α and IL-6 from RAW 264.7 Cells by ZYM

TNF- α and IL-6 levels of RAW 264.7 culture supernatants were measured by ELISA. The final concentration of ZYM were 10, 20 μ g/ml. A culture supernatant after 48 h of culture was collected and the cytokine concentration was measured.

Fig. 3. Vascular Permeability (VP)

A stimulant, such as ZYM, or ZYM treated with serum or plasma, was i.p. injected to mice. Thirty min after the injection, an aqueous solution of Evans blue was injected *via* an i.v. route. Thirty min later, mice were sacrificed and the peritoneal exudated fluids were collected by lavaging with PBS. After centrifugation to remove any debris, the concentration of Evans blue in the peritoneal cavity was measured at 630 nm.

Table 1. Liver and Spleen Weights of ZYM or Serum/Plasma-Treated ZYM

	Liver			Spleen		
	T-Test			T-Test		
	Mean $\pm$ S.D. (g)	To Control	To ZYM	Mean $\pm$ S.D. (mg)	To Control	To ZYM
Control	2.4 $\pm$ 0.3	—	—	135 $\pm$ 25	—	—
ZYM	2.9 $\pm$ 0.2	$p < 0.01$	—	364 $\pm$ 71	$p < 0.01$	—
Serum + ZYM	2.9 $\pm$ 0.2	$p < 0.01$	—	258 $\pm$ 55	$p < 0.01$	$p < 0.05$
Plasma + ZYM	2.8 $\pm$ 0.2	$p < 0.01$	—	247 $\pm$ 66	—	$p < 0.05$

ZYM or serum(plasma)-treated ZYM (1 mg) was injected i.v. to 5-weeks-old mice ($n=5$). Organs were collected 1 week later.

Table 2A. Antitumor Activity of Serum/Plasma-Treated ZYM

No.		Mean $\pm$ S.D. (g)	Inhibition (%)	CR/n	T-Test	
					To control	To ZYM
1	Control	13.1 $\pm$ 7.1	—	0/22	—	—
2	ZYM	3.1 $\pm$ 4.0	76.5	7/22	$p < 0.01$	—
3	Serum + ZYM	8.5 $\pm$ 7.4	35.4	2/7	$p < 0.05$	$p < 0.05$
4	Plasma + ZYM	6.1 $\pm$ 5.8	53.5	2/14	$p < 0.01$	$p < 0.05$

ZYM and serum- or plasma-treated ZYM was administered 3 times (7, 9 and 11 d) 1 week after sarcoma-180 inoculation. Dose of ZYM was 1 mg/mouse/i.v. CR/n = number of complete regression/total.

Table 2B. Antitumor Activity of Serum/Plasma-Treated ZYC

No.		Mean $\pm$ S.D. (g)	Inhibition (%)	CR/n	T-Test	
					To control	
1	Control	8.0 $\pm$ 4.7	—	0/9	—	—
2	ZYC (i.p.)	1.9 $\pm$ 3.0	75.9	2/6	$p < 0.01$	—
3	ZYC (i.v.)	0.5 $\pm$ 1.0	93.7	3/9	$p < 0.01$	—
4	Serum + ZYC (i.p.)	5.0 $\pm$ 5.9	37.9	2/5	—	To No. 6; $p < 0.05$
5	Plasma + ZYC (i.p.)	4.4 $\pm$ 6.4	45.4	0/6	—	—
6	Serum + ZYC (i.v.)	0.7 $\pm$ 1.0	90.9	4/9	$p < 0.01$	—
7	Plasma + ZYC (i.v.)	1.1 $\pm$ 1.7	86.9	5/9	$p < 0.01$	—

ZYC and serum- or plasma-treated glucans were administered 3 times (7, 9 and 11 d) 1 week after sarcoma-180 inoculation. Dose of SPG was 50 μ g/mouse/i.p., ZYC was 250 μ g/mouse/i.p. and 1 mg/mouse/i.v.

Table 2C. Antitumor Activity of Plasma-Treated SSG

No.		Mean $\pm$ S.D. (g)	Inhibition (%)	CR/n	T-Test
					To control
1	Control	4.2 $\pm$ 2.3	—	0/9	—
2	SSG	0.2 $\pm$ 0.2	96.3	4/10	$p < 0.01$
3	Plasma + SSG	0.1 $\pm$ 0.2	98.3	8/11	$p < 0.01$

SSG- and plasma-treated SSG were administered 3 times (7, 9 and 11 d) 1 week after sarcoma-180 inoculation. Dose of SSG was 250 μ g/mouse/i.p.

also be due to the reduced activity of ZYM by protein binding (Table 1).

Antitumor Activity We showed earlier that the antitumor activity of β -glucans such as ZYM, ZYC, SPG, SSG, and GRN were reduced by earlier administration, but β -glucans itself long remained in the liver and spleen for a very long period. To test the contribution of the protein binding on the antitumor activity, we examined the antitumor activity of the ZYM (Table 2A), ZYC (Table 2B) and a soluble β -glucan, SSG (Table 2C), with or without treatment with serum or plasma. The antitumor activity of ZYC was higher than that by ZYM. The antitumor activity of ZYM (i.v.) treated with serum or plasma was significantly reduced. The antitumor activity of ZYC (i.p.) was also reduced, when treated with serum or plasma. However, the antitumor activity of ZYC (i.v.) was only slightly reduced by serum or plasma treatment. The activity of SSG was not reduced when treated with plasma. These facts strongly suggest that protein binding is an important contributing factor in modulating immunopharmacological activity, at least in the case of particulate β -glucans.

DISCUSSION

We have already shown that β -glucans remained in the body for a very long period and, in these organs, the structures of the β -glucans were not significantly modified. Thus, the immunomodulating activity of β -glucan is relatively long lived, especially its antitumor activity to solid form tumors.⁵⁾ The β -glucan preparation, extracted from the liver one month after the β -glucan administration, was still active. However, even in the case of antitumor activity, the activity was not completely retained: a certain part of the activity was lost during the month.¹⁵⁾ In addition, another activities, such as the priming activity to TNF- α synthesis¹⁶⁾ and NO synthesis,¹⁷⁾ disappeared earlier. These facts strongly suggest the presence of a certain inactivation mechanism other than chemical/physical degradation. The data shown in this paper, that serum- or plasma-treated ZYM or ZYC lose or cause the reduction of several kinds of activities, strongly suggests that protein binding is an important contributing factor to the inactivation of the β -glucan.

It is generally thought that a glucan specific receptor(s) is present on a subset of white blood cells, such as macrophages, and that it functions as a signal transducing molecule. The glucan receptor(s) binding site on a glucan might be covered and thus disappear after protein binding; therefore the signal transduction could not proceed after that period. However, except for the complement activation, all of the activities monitored in this paper partially remained after the serum/plasma treatment. These facts also suggest the significant contribution of serum/plasma protein-mediated signal transduction pathways on the immunomodulating effect of ZYM/ZYC. Binding of complement components on the surface of

ZYM is easily confirmed by the capacity to activate a complement by ZYM. Complement receptor mediated signal transduction might be important. We have already shown that plasma clotting was also enhanced by β -glucans and was strongly related to the binding of glucan to the fibrinogen and/or fibronectin.¹⁸⁾ The receptor for these proteins might be also important. In preliminary experiments, we found that several other proteins are bound to ZYM; thus, receptors for these proteins might also contribute to the signal transduction.

Increased vascular permeability (VP) is an important step in subsequent inflammatory and immune reactions because of the increasing chance to come into contact with soluble and/or cellular components in blood. This step is also important to the production of cytokines and to modulating the cytokine network. The reduction of the VP reaction might induce weaker inflammatory and immune responses.

As shown in the introduction, β -glucan once incorporated in the body for *i.e.*, cancer treatment by β -glucan as the biological response modifier or to treat a fungal infection, remains deposited for very long period, especially in the liver and spleen. It is no doubt that the above property is one of the most characteristic features of β -glucan, a representative component of the microbe. Usually, β -glucan has a very high molecular weight, close to 1 million, and is poorly soluble in a physiological buffer. In contrast, many microbial substances are easily degraded to metabolites having a lower molecular weight: peptidoglycan and chitin by lysozyme, an α -glucan by amylase, protein by protease, nucleic acid by nuclease. It is generally thought that the self defense system of the host can clean up all of the invaders by various mechanisms. However, transplanted organs, such as kidney and bone marrow, require immune suppressive therapy to protect against rejection by the host, even though HLA molecules are the only target of the recipient's immune system. Compared to these organs, fungal β -glucan has significantly high xenogenicity, and thus might cause an acute as well as chronic inflammatory/immune response. The data shown in this paper, that only a part of the immune stimulatory response can be neutralized by serum/plasma treatment, well support the above concept. It is also suggested that a host self defense system uses several mechanisms to correspond to and degrade β -glucan.

In addition, we also showed data concerning the plasma treatment of a soluble β -glucan, SSG, and suggested that

the plasma treatment could not diminish the antitumor activity of SSG. As shown in the introduction, a soluble β -glucan, such as SSG, did not produce reactive oxygen species nor arachidonate metabolites from macrophages, probably in relation to the model of engulfing β -glucans, phagocytosis and pinocytosis. These facts strongly suggest that the inactivation mechanism of soluble and insoluble glucans might include some differences.

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