Effect of β -Glucans on the Nitric Oxide Synthesis by Peritoneal Macrophage in Mice

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Nitric oxide (NO) is an important effector molecule on antimicrobial and antitumor effects of macrophages. (1 $\rightarrow$ 3)- β -D-Glucan (β -glucan) is well known to show various immunopharmacological effects such as antimicrobial effect and antitumor effect by activating various points of host defense mechanisms. This paper deals with NO synthetic activity of peritoneal macrophage (PM) induced by β -glucan administration in mice. The activity was determined by measuring NO concentration in PM culture by Griess reagent after 24 or 48 h *in vitro* culture. Administration (i.p. or i.v.) of a branched soluble (1 $\rightarrow$ 3)- β -D-glucan, grifolan (GRN), from *Grifola frondosa* enhanced NO synthesis of PM dose and time dependently. The activity was abrogated by the addition of *N*^G-monomethyl-L-arginine (L-NMMA) *in vitro*. The most significant activity was observed at 3–7 d after the administration of GRN (250 μ g/mouse). PM from all strains of ICR, C3H/HeN, C3H/HeJ, BALB/c, BALB/c nu/nu, C57BL, and AKR mice showed significant activity by GRN administration. Among β -glucans tested, SSG and OL-2, highly branched soluble glucans, and a particulate β -glucan, zymosan, showed similar activity. Addition of GRN directly to *in vitro* RAW 264.7 or proteose peptone induced peritoneal macrophage (PP-PEC) culture could not enhance NO synthesis. However, NO synthesis of PP-PEC was enhanced *in vitro* by addition of GRN in the presence of interferon γ (IFN γ). Gene expression of IFN γ mRNA in the liver and PEC were enhanced in GRN administered mice assessed by reverse transcriptase assisted PCR (RT-PCR) method. These facts strongly suggested that β -glucan has capacity to enhance NO synthesis of PM *in vivo* through IFN γ mediated mechanism.

Key words β -glucan; nitric oxide (NO); macrophage; grifolan (GRN)

It is generally accepted that (1 $\rightarrow$ 3)- β -D-glucans activate host defense systems *in vivo*, and enhance antitumor or antimicrobial activities.¹⁾ Some of the (1 $\rightarrow$ 3)- β -D-glucan, lentinan from *Lentinus edodes* and Schizophyllan (SPG) from *Schizophyllum commune*, have been used clinically in Japan.²⁾ There are many studies on antitumor and immunomodulating activities of the (1 $\rightarrow$ 3)- β -D-glucan, such as macrophage functions, NK cell activation, delayed type hypersensitivity (DTH) activation, antibody production, hematopoietic response, blood coagulation system, complement system, and so on.³⁾ β -Glucan shows a broad spectrum of immunopharmacological activity, thus much more study is required to establish a molecular understanding of the biological activities of the β -glucan.

Macrophages are known to play a central role in generation of specific and non-specific immunity.⁴⁾ A certain glucan receptor protein for the particulate glucan was suggested to be displayed on the macrophage surface.⁵⁾ Administration of (1 $\rightarrow$ 3)- β -D-glucan activates macrophages to show cytostatic and cytotoxic activity to the tumor cells.⁶⁾

It has recently been realized that nitric oxide (NO) plays important roles in the body.⁷⁾ NO synthase in nerve system or endothelial cells is constitutive type (cNOS), while macrophages possess an inducible type of NO synthase (iNOS). Macrophages activated by cytokines or lipopolysaccharide (LPS) secrete NO which is toxic to tumor cells and microorganisms.⁸⁾

To elucidate the mechanism of biological activity of the β -glucans under the molecular level, it is therefore important to determine the NO production by macrophage of the glucan administered mice. We have been intensively

studying the structure–activity relationship of the β -glucan mediated biological activities.⁹⁾ In this study, we examine the effect of the (1 $\rightarrow$ 3)- β -D-glucan, grifolan (GRN) obtained from *Grifola frondosa* and several related glucans on the production of NO by peritoneal macrophages (PM) in mice.

MATERIALS AND METHODS

Animals Specific-pathogen-free male ICR mice were purchased from Japan Slc, Inc., Hamamatsu, Japan and used at 6–8 weeks of age. The animals were bred under specific-pathogen-free conditions.

Reagents GRN obtained from liquid-cultured mycelium of *Grifola frondosa* was prepared by the procedure described previously.¹⁰⁾ SSG was from the culture filtrate of *Sclerotinia sclerotiorum*.¹¹⁾ OL-2 was from *Omphalia lapidescente*.¹²⁾ SPG was generously provided from Kaken Pharmaceutical Co., (Tokyo, Japan). CRD was from Wako Pure Chemicals (Osaka, Japan). Zymosan A (zymosan, ZYM) was purchased from Sigma Chemical Co., (St. Louis, MO). RPMI 1640 medium (Nissui Seiyaku Co., Tokyo) supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), and 5 mM HEPES (Sigma) was used for cell culture in this paper. Recombinant murine interferon γ (IFN γ) was purchased from Genzyme Co.

Macrophages Cells were collected from the peritoneal cavities of mice by washing twice with 5 ml of Hank's balanced salt solution (HBSS) containing heparin (5 U/ml). The cells were washed twice and resuspended in RPMI 1640 medium. The total cell number was counted with a hemocytometer, and peritoneal cells were inoculated

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into flat-bottomed 24, 48, or 96-well tissue culture plates (1×10^6 cells/ml) in RPMI 1640 medium containing 10% fetal calf serum (FCS). After incubation for 2 h at 37 °C in a CO₂ incubator, nonadherent cells were removed from some of the cells by washing with RPMI 1640 medium. The cell suspensions were incubated for 24 or 48 h at 37 °C in a CO₂ incubator.

Measurement of NO Production by PM NO, determined by the accumulation of nitrite in the culture medium, was measured spectrophotometrically using the Griess reaction with sodium nitrite as the standard.¹³⁾ Briefly, 100 μ l of culture supernatant was mixed with 100 μ l of 1% sulfanilamide and 0.15% *N*-1-naphthylethylenediamine dihydrochloride in 2.5% H₃PO₄. After 10 min at room temperature, optical density was measured at 550 nm.

Reverse Transcriptase Assisted PCR (RT-PCR) Total cellular RNA was extracted from the entire peritoneal exudated cells (PEC) and approximately 30 mm³ of each liver and spleen by the acid guanidium thiocyanate/phenol/chloroform extraction method, then resuspended in 20 μ l of diethyl pyrocarbonate (DEPC)-treated water and stored at -80 °C until subjected to cDNA preparation. cDNA was prepared using random hexamer and Moloney-Murine leukemia virus reverse transcriptase (MMLV RTase)(United States Biochemical Corporation). Samples were stored at -80 °C until subjected to PCR amplification. Aliquots of cDNA were amplified in 500 μ l microcentrifuge tubes in the presence of 0.15 μ M sense primer, 0.15 μ M antisense primer, and 0.5 units of Taq DNA polymerase in PCR buffer. The reaction mixture was overlaid with 3 drops of mineral oil, and PCR was performed in a Perkin-Elmer Cetus DNA Thermal Cycler (Connecticut U.S.A.) for 35 or 40 cycles, each cycle consisting of 1 min of denaturation at 94 °C, 2 min of annealing at 55 °C, and 3 min of extension at 74 °C. The reaction product was visualized by electrophoresis of 10 μ l of the reaction mixture at 50 V for 40 min in a 1.5% agarose gel containing 0.5 μ g of ethidium bromide per ml.

The primers used for this experiment were as follows:

β -actin	sense primer	(s) 5'-CCATGGATGACGATATCGCT-3'
	antisense primer	(a) 5'-TCATGAGGTAGTCTGTCAGGT-3'
IFN γ	sense primer	(s) 5'-CGCTACACACTGCATCTTGG-3'
	antisense primer	(a) 5'-GGCTGGATTCCGGCAACA-3'

Statistics Statistical evaluations in all experiments were performed by Student's *t*-test. A value of $p < 0.05$ was considered significant.

RESULTS

NO Production by PM from GRN-Administered Mice

To examine the *in vivo* activation of macrophage to produce NO by administration of GRN, macrophage (PM) was collected from the peritoneal cavity of GRN administered mice and cultured *in vitro* for 24 to 48 h. The supernatant was used for NO determination using Griess reagent with NaNO₂ as a reference. The results of kinetics study on the NO production by PM obtained from mice intraperitoneally (i.p.) administered GRN are shown in Fig. 1. When mice were injected with 250 μ g/mouse of GRN, a significant activity of NO production was observed in PM as early as 24 h after administration, and thereafter gradually increased. The activity remained high for at least 2 weeks. Similar kinetics of NO synthetic activity was observed by intravenous administration of GRN (data not shown).

Figure 2 shows a dose-dependency of i.p. administered GRN on the NO production by PM. NO production was enhanced by the administration of a wide range of doses (50–2500 μ g/mouse). Intravenous administration of GRN showed similar dose response (data not shown).

To confirm the accumulation of NO in culture supernatant due to the activity of NO synthase, the NO synthetase inhibitor, *N*^G-monomethyl-L-arginine (L-NMMA) was added to the culture. As shown in Fig. 3, GRN-induced NO synthesis was significantly inhibited by L-NMMA, strongly suggesting that the inducible NO

Fig. 1. Kinetics of GRN (i.p.)-Induced NO Synthesis by PM in Mice

GRN (250 μ g/mouse) or saline was i.p. administered to male ICR mice (6 weeks old) on days -13, -8, -5, -3, -2, -1, or -0.5. Mice were sacrificed on day 0 and peritoneal cells were collected. Each 500 μ l of 1×10^6 /ml PEC in RPMI 1640 medium was placed on a 48 well plate and incubated in a 5% CO₂ incubator. Two hours later, nonadherent cells were removed by washing with RPMI 1640 medium. FCS was added to the final concentration of 10%. NO concentration in the culture medium was measured after 48 h of culture by Griess reagents. Values represent mean $\pm$ S.D. of 3 mice.

Fig. 2. Dose Dependency of GRN Induced NO Synthesis of PM in Mice

GRN (50, 100, 250, 500, 1000, 2500 µg/mouse) or saline was i.p. administered to male ICR mice (6 weeks old) on day 0. Mice were sacrificed on day 4 and peritoneal cells were collected. Other conditions were as described in the legend to Fig. 1.

Fig. 3. Effect of L-NMMA Treatment *in Vitro* on GRN-Induced NO Synthesis of PM in Mice

GRN (250 or 750 µg/mouse) was i.p. administered to male ICR (6 weeks old) on day 0. Mice were sacrificed on day 4 and peritoneal cells were collected. Ten µl of several concentrations of L-NMMA was placed on 96 well plate (3 wells each). Two hundred µl of 1×10^6 /ml peritoneal cells in RPMI 1640 medium in 10% FCS was placed on the above wells and incubated in a 5% CO₂ incubator. NO concentration in the culture medium was measured after 48 h of culture by Griess reagent.

synthase was activated by GRN administration. The fact that L-NMMA did not inhibit the mitogenic response of spleen cells to bacterial LPS or concanavalin A strongly suggested the absence of direct cytotoxicity of L-NMMA to macrophage (data not shown).

Effect of Various Glucans on NO Production of PM Figure 4 shows the effect of i.v. administered ZYM on the NO production of PM. The data strongly suggested that the effective dose was much narrower (from 250 to 2000 µg/mouse of ZYM) than GRN. It is also notable that significant activity was observed as early as 24 h after administration of ZYM (1000 µg/mouse, data not shown).

Table 1 shows the effect of the various glucans on NO production. Indicated glucans were administered by i.p. route and the activity was measured 4 d after the ad-

ministration. In addition to GRN, the highly branched glucans SSG and OL-2 showed strong activity. SPG, a glucan having triple helix conformation, could not induce NO synthesis.

GRN-Induced NO Production in Various Strains of Mice It is well known that immunopharmacological activities of biological response modifiers (BRM) are influenced by the genetic background of mice. To screen the genetic background influencing NO synthesis by GRN, we compared the activity in several strains of mice. As shown in Table 2, the activity was induced by GRN in strains tested. Activation of PM by GRN in nu/nu mice suggested independence of T cell activation. Activation of PM by GRN in C3H/HeJ mice also strongly suggested that the mechanisms were different from that by bacterial LPS. Activation of PM by GRN in the AKR strain, C5 deficiency, is strongly related to the lower contribution of the complement cascade.

Mechanism of GRN-Induced NO Production *in Vivo* As shown above, NO synthesis of PM required at least 24 h following GRN administration. We have also found that the activity could not be induced by the addition of GRN to the macrophage-like cell line RAW 264.7 *in vitro* (data not shown). Similar results were shown using proteose peptone elicited macrophage (PP-PEC) cultures *in vitro* (Table 3); however, NO was induced in a PP-PEC culture by the addition of GRN in the presence of IFN γ (Table 3).

On the other hand, using RT-PCR, IFN γ mRNA was observed in liver and PM of GRN administered mice (Fig. 5). These facts strongly suggested that activation of NO synthesis in macrophages by GRN *in vivo* is mediated by direct activation of IFN γ activated macrophage by GRN.

DISCUSSION

In this paper, we demonstrated that administration of the (1 $\rightarrow$ 3)- β -D-glucans enhanced NO production by PM in mice. Both soluble glucan (GRN, SSG, OL-2) and insoluble glucan (ZYM) possessed this activity, suggesting

Fig. 4. Effect of ZYM (i.v.) on NO Synthesis of PEC in Mice

ZYM or Saline was i.v. administered to ICR mice (male, 6 weeks old). Mice were sacrificed on day 4 and peritoneal cells were collected. Other conditions were as described in the legend to Fig. 1.

Table 1. Effect of Various β -Glucans on NO Synthesis of PEC in Mice

	Saline	CRD	GRN	SPG	SSG	OL-2
NO conc. (μ M)	3.1 $\pm$ 0.7	3.0 $\pm$ 0.5	37.6 $\pm$ 25.3	5.8 $\pm$ 1.5	31.5 $\pm$ 2.2	64.2 $\pm$ 3.4
t-Test to saline		ns	$p < 0.001$	ns	$p < 0.001$	$p < 0.001$

β -Glucans were i.p. administered to male ICR mice (6 weeks old) on day 0. Mice were sacrificed on day 4 and PEC were collected. Other conditions were as described in the legend to Fig. 1.

Table 2. Effect of GRN on NO Synthesis of PEC in Various Strains of Mice

Mouse strain	NO conc. (μ M)	
	Saline (n=2)	GRN (n=3)
ICR	3.1 $\pm$ 0.7	37.6 $\pm$ 25.3
C3H/HeN	2.1 $\pm$ 0.5	25.6 $\pm$ 20.5
C3H/HeJ	2.7 $\pm$ 1.7	11.7 $\pm$ 6.3
BALB/c	1.3 $\pm$ 1.2	26.8 $\pm$ 0.3
BALB/cnu/nu	13.5 $\pm$ 1.1	25.5 $\pm$ 4.5
C57BL	1.2 $\pm$ 0.3	7.9 $\pm$ 2.1
AKR	2.1 $\pm$ 9.5	41.6 $\pm$ 5.1

GRN (250 μ g/mouse) or saline was i.p. administered to various strains of male mice (6 weeks old) on day 0. Other conditions were as described in the legend to Fig. 1.

Table 3. Synergistic Effect of GRN and IFN γ on NO Synthesis of PM *in Vitro*

	+ Medium	+ IFN γ 10 U/ml
Medium	2.79 $\pm$ 0.65	4.37 $\pm$ 0.94
GRN 25 μ g/ml	4.06 $\pm$ 0.27***	9.68 $\pm$ 1.69***
GRN 100 μ g/ml	4.66 $\pm$ 0.81***	14.99 $\pm$ 2.60***

Protease peptone (2 ml) was i.p. administered to C3H/HeJ mice on day 0. Mice were sacrificed on day 3 and PEC were collected. Cells were placed on a 96 well-plate at 2×10^6 /well in 200 μ l of RPMI 1640 medium containing 10% FCS and incubated in a humidified 5% CO $_2$ incubator at 37 $^{\circ}$ C. Two hours later, non-adherent cells were removed by washing with RPMI 1640 medium and GRN and/or IFN γ were added. NO concentration in the culture supernatant was determined after 24 h of culture by Griess reagent. Values represent mean $\pm$ S.D. *** $p < 0.001$.

Fig. 5. Gene Expression of IFN γ mRNA in Liver, Spleen and Peritoneal Cells by i.p. Administration of GRN (250 μ g/mouse) to ICR Mice (2 Mice Each)

Three days after the administration, liver (LIV), spleen (SPL) and peritoneal cells (PEC) were collected and immediately frozen to prepare total RNA. RT-PCR analysis was performed as described in Materials and Methods.

the common property of immunomodulating β -glucans. We have studied the antitumor activities and structure-function relationships of (1 $\rightarrow$ 3)- β -D-glucans.¹⁴⁾ GRN is a gel-forming (1 $\rightarrow$ 6)-branched (1 $\rightarrow$ 3)- β -D-glucan having a branch at every third main chain unit (degree of branching (DB)=2/6). It is interesting to investigate NO production of macrophages by (1 $\rightarrow$ 3)- β -D-glucans having different degrees of branching. We have found that the higher branched glucans (SSG (DB=3/6), OL-2 (DB=4/6)) also induced NO synthesis. We have already shown that the antitumor activity of OL-2 to the solid form of Sarcoma 180 was less than that of GRN and SSG. This strongly suggests that the structure-activity relationship of these two activities differs, at least part. It is thought that NO production by the (1 $\rightarrow$ 3)- β -D-glucans would be to some degree related to the broad spectrum of the β -glucan mediated activities.

It is suggested that gel producing (1 $\rightarrow$ 3)- β -D-glucans possess at least two conformations: a triple helix conformer and a single helix conformer. Although it may be that the triple helix is necessary for the antitumor activity, structure-function relationships of antitumor (1 $\rightarrow$ 3)- β -D-glucans are still controversial.¹⁵⁾ Therefore, the study of structure-activity relationships is important, especially in relation to the ultrastructure for NO production by macrophages *in vivo*. We have very recently found that the sodium hydroxide treatment of SPG (SPG-OH)

effectively changed the conformer to induce NO from macrophages of glucan administered mice.¹⁶⁾ This implies strongly that the single helical conformation is significantly important for NO synthesis *in vivo*.

Ding *et al.*¹³⁾ studied NO production of PM by twelve kinds of cytokines *in vitro*, and only IFN γ induced NO production. We have shown that the glucans used in this paper could not or could hardly induce NO production by PM *in vitro* (data not shown), and that the activation was observed in the presence of IFN γ (Table 3). We have also found the gene expression of IFN γ mRNA in the liver and PEC of GRN administered mice. Generally IFN γ is secreted by TH1 cells or NK cells. As the antigen presentation has not been induced by this assay system, TH1 cells probably could not be activated in this experiment. Thus, we have suggested that β -glucan mediated enhancement of NO synthesis by PM is due to the activation of macrophages by IFN γ from NK cells, and the following direct activation of macrophages by β -glucans.

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