Enhancement of Cytokine Production by Macrophages Stimulated with (1→3)-β-D-Glucan, Grifolan (GRN), Isolated from *Grifola frondosa*

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The ability of grifolan (GRN), a purified fungal (1→3)-β-D-glucan, to induce various cytokines from macrophages was examined *in vitro*. Interleukin-6 (IL-6) activity in supernatants from the culture of macrophage cell line, RAW264.7 was dependent on increasing doses of GRN. The level of IL-6 induced with 500 μg/ml of GRN was comparable to that induced with lipopolysaccharide (LPS) 10 μg/ml. Enhancement of the mRNA level of IL-6 by treatment with GRN was detected by reverse transcriptase-polymerase chain reaction (RT-PCR). The effect of GRN on production of IL-6 was also observed using peritoneal macrophages from C3H/HeJ mice which did not respond to endotoxins. This data suggested that the ability of GRN to activate IL-6 production of macrophages is not due to contamination of endotoxins in the preparation. Enhanced production of cytokine by GRN was observed not only with IL-6, but also with interleukin-1 (IL-1) and tumor necrosis factor α (TNFα). In the production of TNFα, GRN was more effective than LPS used in this study. Other soluble or gel-forming(1→3)-β-D-glucans from various sources did not enhance the production of such cytokines although they are structurally similar to GRN. The above results indicated that GRN is a novel macrophage activator which augments cytokine production without dependence on endotoxins.

Keywords (1→3)-β-D-glucan; cytokine; macrophage; inflammatory cytokine; RAW264.7

(1→3)-β-D-Glucans are known to possess immunomobulating effects, and two of them, lentinan (from *Lentinus edodes*)[1] and sonifilan (SPG, from *Schizophyllum commune*)[2] are used clinically in Japan. Recently, we isolated from the first time grifolan (GRN), a gel-forming (1→6)-branched (1→3)-β-D-glucan, from liquid-cultured mycelium of *Grifola frondosa* (Table I). The primary structure of GRN is similar to SPG (originally called schizophyllan), consisting of a β-(1→3)-polyglucose backbone, with a third residue of 6-*O*-substituted by mono-glucosyl branches (M_r=500000) (Fig. 1). GRN possesses a gel structure consisting of a single or triple helical (1→3)-β-D-glucosyl backbone.[3,4] In a previous report describing the ability of various β-glucans to generate H_2O_2 from peritoneal macrophages, particulate β-glucans such as yeast glucan was able to stimulate the H_2O_2 production significantly, whereas gel-forming β-glucans including GRN, lentinan, SPG, and *Sclerotinia sclerotiorum* IFO 9395 glucan (SSG) had no triggering effect on the macrophages *in vitro*.[5] These results indicated that the activation pathway in peritoneal macrophages stimulated with particulate β-glucans was not identical to gel-forming β-glucans, and implied that each activation pathway had not yet been fully elucidated. We have also reported that GRN administered *in vivo* enhanced immunoreactivity, including activation of macrophages, cytotoxic T cells and NK cells.[6–9] Such cellular activation induced by (1→3)-β-D-glucans are thought to be caused by both quantitative and qualitative alterations because the glucans enhanced haematopoietic cell growth and augmented lymphocyte function.[10,11] In these processes, various cytokines released from macrophages/monocytes stimulated with (1→3)-β-D-glucan may play important roles in the initial activation. Macrophages have a fundamental protective function of ingesting and killing invading organisms, and release a vast number of factors involved in host defense and inflammation.[12] Moreover, they are known to play critical roles in the induction, regulation, and expression of both humoral and cellular immune responses. For instance, macrophages activated by lipopolysaccharide (LPS) release several mediators, including inflammatory cytokines (IL-1, IL-6, interleukin-8 (IL-8), granulocyte-clony stimulating factor (G-CSF) and tumor necrosis factor α (TNFα)), prostaglandins and nitric oxide, and these mediators induce activation and differentiation of lymphocytes, and proliferation of

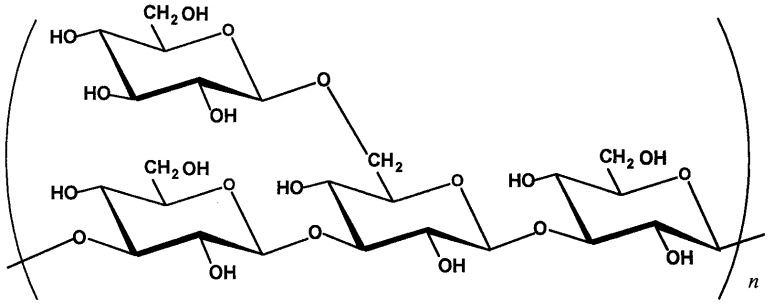

Fig. 1. Primary Stucture of GRN

granulocytes, support enhanced cytotoxicity against tumor cells and accelerate immunoreactivity *in vivo*. (1→3)-β-D-Glucans or GRN may activate immune responses in the same way.[13,14]

In this study, we found *in vitro* that GRN augmented production of a pleiotropic cytokine, IL-6. Since IL-6 exhibits a broad spectrum of immunoregulatory activities as diverse as B cell differentiation, induction of interleukin-2 (IL-2) production in T cells, and induction of acute phase proteins in hepatocytes,[15,16] we considered that augmented production of IL-6 by GRN should activate host-defense systems.

In further investigation, other gel-forming (1→3)-β-D-glucans, SPG and SSG with anti-tumor activity *in vivo*[2,10] were tested for their ability to augment IL-6 production by macrophages *in vitro*.

The present study concerned 1) the ability of GRN to induce IL-6 release from macrophages *in vitro*, 2) difference in effects on cytokine release between GRN and LPS, and 3) comparison of potentials as cytokine inducers among several soluble and gel-forming (1→3)-β-D-glucans.

MATERIALS AND METHODS

Preparation of (1→3)-β-D-Glucans and LPS GRN was prepared from liquid-cultured mycelium of *Grifola frondosa* as described previously.[3,4] Briefly, *G. frondosa* var. *Tokachiana* was grown in 100 ml of medium containing glucose (2.0%), polypeptone (0.6%), cane sugar (2.0%) and soybean oil (0.1%), pH 4.5, with reciprocal shaking at 25 °C for 14 d. The mycelium was separated from the filtrate by filtration, and was incubated in 0.5% citrate buffer (pH 4.0) containing 5% glucose at 25 °C. After incubation for 6 d, the mixture was centrifuged and the supernatant solution was diluted with 1 volume of ethanol. An aqueous solution of the resulting precipitate was diluted with 1 volume of ethanol and the precipitate was dissolved in 8 M urea. The solution was further purified by DEAE-Sephadex A-25 (Pharmacia) to remove contaminating proteins. The non-adsorbed fraction was dialyzed against water and precipitated with ethanol. The precipitate was dried *in vacuo* and used as GRN. Contamination of LPS in this preparation was less than 0.0001% (1.0 pg/mg of test sample; determined by a chromogenic endotoxin specific assay, Endospecy™, Seikagaku Kogyo Co., Tokyo). SPG was generously provided by Kaken Pharmaceutical Co., Ltd. (Tokyo). SSG was prepared by the method described previously.[17,18] Laminarin (LAM) and dextran (DEX) were purchased from Sigma Chemical Co., St. Louis, MO and Seikagaku Kogyo Co., Tokyo, respectively. LPS used in this study was repurified from commercially available LPS obtained from *Escherichia coli* 0127: B8 (Sigma Chemical Co.) by the Westphal method.[19]

Preparation of Macrophages Peritoneal macrophages were isolated from C3H/HeJ mice, 6 to 8 weeks old, which had been injected intraperitoneally with 2 ml of proteo-speptone 3 d prior to peritoneal lavage with 10 ml of Hanks' balanced salt solution (Nissui Pharmacuetical Co., Ltd., Tokyo, Japan) containing 5 U/ml heparin. Collected cells were washed twice with RPMI1640 and then cultured in RPMI1640 containing 1% fetal calf serum (FCS) at a density of 2.2×10^6 cells/ml. Cells were allowed to adhere for 3 h to a 24-well culture flask at 37 °C in a 5% CO_2 incubator. Then, cultures were washed twice with RPMI1640 to remove nonadherent cells prior to the addition of 1 ml of fresh RPMI1640 containing 10% FCS (PRMI1640/10% FCS) and various concentrations of GRN and LPS. Mouse macrophage-like cells, RAW264.7 (RIKEN Cell Bank, Tsukuba) were cultured to confluence in RPMI1640/10% FCS. RAW264.7 cells were established from the ascite of a tumor induced in a mouse by the intraperitoneal injection of Abelson-leukemia virus; these cells have a capacity for phagocytosis of zymosan and latex beads as can be seen in mouse peritoneal macrophages.[20] RAW264.7 cells were suspended at a density of 2.5×10^6 cells/ml in RPMI1640/10% FCS. Peritoneal macrophages and RAW264.7 cells were stimulated with or without GRN (100, 500 μg/ml) or LPS (10 μg/ml) at 37 °C for 24 to 48 h in a humidified 5% CO_2 incubator. At the end of the incubation, culture supernatant was collected by centrifugation at 300 ***g*** for 5 min. Then, macrophages adhering to the culture plate were lysed in distilled water by repeated freezing and thawing (3 times) followed by addition of a 2-fold concentration of RPMI1640 and filtration through a syringe filter unit (0.20 μm, Corning).

Assay for IL-6 Production IL-6 in the culture supernatant was measured by bioassay using a IL-6 dependent murine hybridoma clone, MH-60/BSF-2, kindly provided by Dr. T. Matsuda (Osaka University, Japan).[21] MH-60/BSF-2 was maintained in RPMI1640/10% FCS containing 200 pg/ml recombinant mouse-IL-6 (rmIL-6: R&D Systems, Minneapolis, MN). For measurement of IL-6 activity, MH-60/BSF-2 was incubated in 96-well plates (1.0×10^4 cells/well) with serially diluted samples obtained from culture supernatant of macrophages. After 42 h of incubation, MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide: DOJIN, Kumamoto, Japan) was added at a concentration of 100 μg/well. Formazan was harvested 6 h later and dissolved in 150 μl of 0.04 N HCl–isopropanol solution. Absorbance of the formazan in each well was measured by a microplate reader (MTP32, Corona Electric, Tokyo) using a 550 nm filter. The data was calculated from the standard curve of recombinant mouse IL-6 (0 to 1 ng/ml).[22]

Assay for IL-1 Production by Thymocyte Proliferation IL-1 production in the culture supernatants and the macrophage cell lysates was measured by a standard assay measuring lymphocyte-activating factor activity in terms of the thymocyte proliferation response.[23] Briefly, a single cell suspension of thymocytes (2.0×10^6 cells/well) taken from C3H/HeJ mice was prepared and added to 96-well flat-bottomed tissue culture plates. Samples, culture supernatants (50 μl) and cell lysates (50 μl) of RAW264.7 cells were diluted and then added to the cell suspension with or without rabbit polyclonal anti-mouse IL-1α serum (Genzyme Co., Boston, MA). The cultures were then incubated in the presence of a submitogenic concentration (2 mg/ml) of phytohemagglutinin-P at 37 °C

for 72 h in a CO_2 incubator. These cultures were pulse labeled with [^{3}H]thymidine (0.5 μCi/well) for the final 12 h of the incubation period. At the end of this period, the cells were harvested and the radioactivity was measured in a liquid scintillation counter. The data were expressed as the mean cpm of triplicate cultures.

Determination of IL-1α Production by ELISA[24] For the ELISA system, a 96-well plate (MS 8596-F, Sumitomo Bakelite Co., Tokyo) was coated with hamster anti-mouse IL-1α monoclonal antibody (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, culture supernatant or cell lysate in duplicate at 37 °C for 40 min and then exposed to rabbit polycolonal anti-mouse IL-1α serum (Genzyme Co., Boston, MA). The plate was developed using peroxidase-labeled goat anti-rabbit IgG (Organon Teknika Co., West Chester, PA) and peroxidase substrate (TMB microwell peroxidase substrate system; Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). Aliquots of recombinant mouse IL-1α (Genzyme Co., Boston, MA) dissolved in 50 μl of BPBST were used to construct a standard curve.

Assay for TNFα Production[25] TNFα in the culture supernatant was measured by a double-sandwich ELISA. This method is identical to ELISA for mouse IL-1α except that capture-monoclonal antibody for mouse TNFα (Pharmingen) and second polyclonal anti-mouse TNFα (Genzyme Co., Boston, MA) were used.

Detection of IL-6 mRNA by Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)[11] Total cellular RNA was isolated from the monolayer of RAW264.7 cells in a 24 well-culture plate by the method of acid guanidium thiocyanate/phenol/chloroform extraction. RNA was reverse transcribed into cDNA as follows: an aliquot of RNA was mixed with a master mix containing a buffer for Maloney murine leukemia virus-reverse transcriptase (MMLV RTase), 2′-deoxynucleoside 5′-triphosphates (dNTPs), ribonuclease (RNase) inhibitor (8 units), reverse transcriptase from MMLV (40 units), and random hexamers (final 2.5 mM), and incubated at 37 °C for 60 min, at 99 °C for 5 min, and then at 5 °C until use. An aliquot of cDNA was mixed with $MgCl_2$ (final 2 mM), a PCR buffer, Taq DNA polymerase (0.5 units), and a set of primers (see below), and 30 cycles of PCR reaction (step 1, 1 min at 94 °C; step 2, 2 min at 55 °C; step 3, 3 min at 74 °C) were performed in a Perkin–Elmer Cetus DNA thermal cycler to amplify DNA. The resulting DNA fragment was detected by agarose gel electrophoresis after staining with ethidium bromide. β-Actin mRNA was used as a control and the results were compared under the same concentration of β-actin mRNA. Primers used for this experiment were β-actin (sense [s], 5′-GCCATGGATGACGATATCGCT-3′; antisense [a], 5′-TCATGAGGTAGTCTGTC AGGT-3′); IL-6 ([s], 5′-TTCCTCTCTGCAAGAGACT-3′; [a], 5′-TGTATCTCTCTGAAGGACT-3′).

RESULTS

Effect of GRN on IL-6 Production by Macrophages To investigate the effect on IL-6 production from macrophages stimulated with GRN, RAW264.7 cells were cultured with 100 and 500 μg/ml of GRN at 37 °C for 24 h. As a positive control, LPS was simultaneously cultured with RAW264.7 cells. The culture supernatant from GRN-treated RAW264.7 cells showed a significant amount of IL-6 comparable to that from LPS-treated cultures (Fig. 2). A similar result was obtained in an experiment using peritoneal macrophages from ICR mice (data not shown). Since contamination of LPS in GRN was less than 0.0001% (Table I), it would have negligible

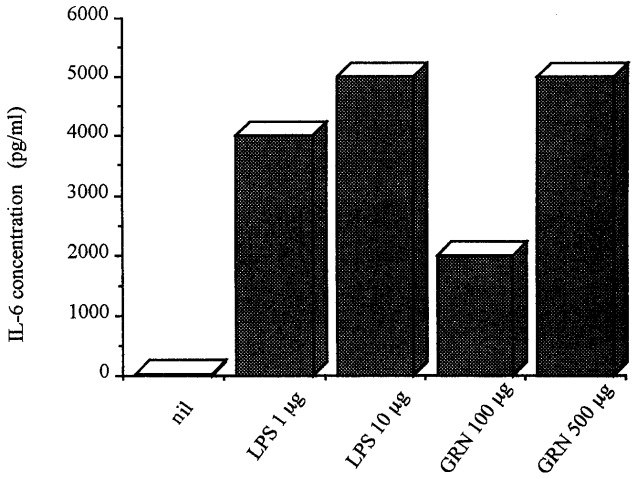

Fig. 2. IL-6 Production by RAW 264.7 Cells Stimulated with GRN and LPS

RAW 264.7 cells were stimulated with or without GRN (100, 500 μg/ml) and LPS (10 μg/ml) for 24 h at 37 °C in a humidified 5% CO_2 incubator. IL-6 activities in the culture supernatants from RAW 264.7 cell were measured as described in Materials and Methods.

TABLE I. Physicochemical Properties of GRN

Sample	Yield[a] (%)	Sugar[b] (%)	Protein[c] (%)	Endotoxin content[d] (%)	$[\alpha]_D$[e]
GRN	30.0	91.3	0.3	<0.0001	−6.4°

a) From 100 g of lyophilized mycelium of *Grifola frondosa*. *b*) Determined by phenol–sulfuric acid method. *c*) Determined by Lowly–Folin method. *d*) Determined by endospecy limulus test. *e*) $[\alpha]_D$ in 0.3 N NaOH solution.

TABLE II. IL-6 and IL-1α Production by Peritoneal Macrophages from C3H/HeJ Mice Stimulated with GRN and LPS

Cell culture treatment (μg/ml)		IL-1α (pg/ml)	IL-6 (ng/ml)
Nil		9.44	0.111
GRN	100	19.62	0.642
	500	137.02	20.182
LPS	1.0	0.00	0.107
	10.0	13.87	0.172

Peritoneal macrophages (PMs) were collected from C3H/HeJ mice that had been treated with proteospeptone and had adhered to a 24-well culture flask (1.1×10^6 PMs/well). And PMs were stimulated with or without GRN (100, 500 μg/ml) and LPS (10 μg/ml) for 24 h at 37 °C in a humidified 5% CO_2 incubator. IL-6 activities in the culture supernatants and IL-1α activities in cell lysates from PMs were measured as described in Materials and Methods.

influence on the enhancing effect of GRN on IL-6 production. The effect of GRN on IL-6 production was further tested with peritoneal macrophages from C3H/HeJ which is non-responsive to LPS.[26,27] As shown in Table II, 500 μg/ml of GRN was able to augment IL-6 production in supernatant of macrophages from C3H/HeJ, but LPS was not. Moreover, GRN induced IL-1α production in cell lysate by macrophages from C3H/HeJ. These results clearly showed that GRN itself could stimulate macrophages to produce IL-6 *in vitro* in a dose-dependent manner.

Kinetics of IL-6 Translation and Transcription by GRN-Stimulated RAW264.7 Cells We next examined the time course of IL-6 production induced by GRN-treated macrophages. RAW264.7 cells were cultured with 100 and 500 μg/ml of GRN or 10 μg/ml of LPS at 37 °C for 0 to 24 h, and then the IL-6 activity in the culture supernatant was monitored. A significant level of IL-6 was found at 5 h after addition of GRN or LPS, and this reached a plateau at 15—24 h (Fig. 3). This level of IL-6 production was maintained fro at least 48 h (data not shown). No significant differences between 500 μg/ml of GRN and 10 μg/ml of LPS were observed with respect to the level of IL-6 released in the cultures. For a more detailed examination of the time course of IL-6 production, the RT-PCR system was applied to detect mRNA coding IL-6.

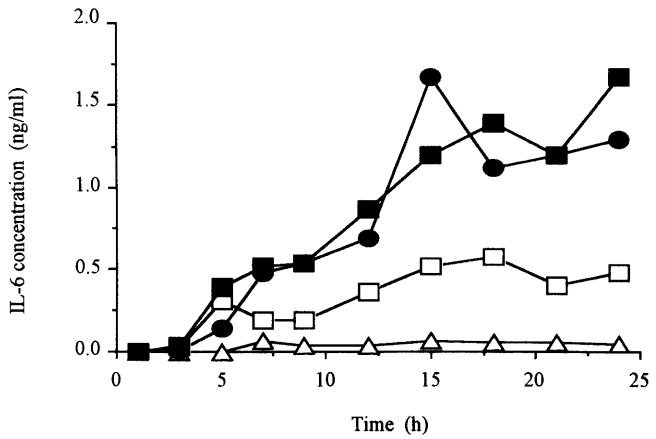

Fig. 3. Kinetics of IL-6 Production by RAW 264.7 Stimulated with GRN and LPS

RAW 264.7 cells were stimulated with GRN 100 μg/ml (□), 500 μg/ml (■), LPS 10 μg/ml (●) or were unstimulated (△) for 24 h at 37 °C in a humidified 5% CO_2 incubator. IL-6 activities in the culture supernatants from RAW 264.7 cells were measured as described in Materials and Methods.

The macrophages were cultured with 500 μg/ml of GRN and 10 μg/ml of LPS at 37 °C for 0.5, 1, 3, 6, and 12 h. The RNAs were then extracted from the stimulated macrophages and were subjected to the RT-PCR system. As can be seen in Fig. 4, IL-6 mRNA expression induced with LPS-treatment was detected after 0.5 h of incubation, and reached maximum steady-state levels after 3 h of incubation, while mRNA induced by GRN-treatment was first detected after 1 h of incubation.

Effect of GRN on Production of IL-1 and TNFα by RAW264.7 Cells We investigated whether GRN activated RAW264.7 cells to produce other inflammatory cytokines such as IL-1 and TNFα. First, IL-1 activity in culture supernatants and cell lysates was assessed by the thymocyte proliferation assay (Table III). By addition of anti-IL-1α neutralization antibody to the samples, the IL-1 activities in both culture supernatants and cell lysates were completely stopped suggesting that GRN and LPS could not augment IL-1β production of RAW264.7 (Table III). Kinetics of IL-1α production in culture supernatants and cell lysates from GRN- and LPS-treated RAW264.7 cells were further investigated by ELISA. As shown in Fig. 5, IL-1α production in cell lysates reached steady-state levels for at least 6 h and declined after 15 h of incubation. In contrast to the cell lysates, IL-1α in culture spernatants increased after 18 h of incubation. Similar levels of IL-1α were observed in LPS-treated RAW264.7 cells, and IL-1α release in culture supernatants was increased after 18 h of incubation. However, treatment with 500 μg/ml of GRN did not stimulate IL-1α secretion in culture supernatants, that higher concentrations of GRN might modulate certain mechanisms related to IL-1α secretion by RAW264.7.

TNFα production by GRN- or LPS-treatment of

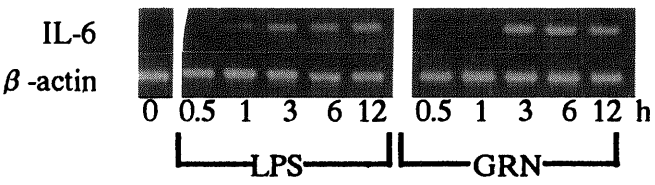

Fig. 4. Expression of IL-6 mRNA in RAW 264.7 Stimulated with GRN and LPS

Total-RNA were isolated from the monolayer of RAW264.7 cells stimulated with or without GRN 500 μg/ml or LPS 10 μg/ml for 0—12 h at 37 °C in a humidified 5% CO_2 incubator in a 24-well culture flask, and expression of IL-6 mRNA was assessed by RT-PCR methods as described in Materials and Methods.

TABLE III. Effect of IL-1α Neutralization on IL-1 Production by RAW264.7 Stimulated with GRN and LPS

Cell culture treatment (μg/ml)		IL-1 activity (cpm ± S.D.)			
		Cell lysate		Supernatant	
		Anti-IL-1α antibody treatment			
		−	+	−	+
Nil		2936.0 ± 197.0	1938.0 ± 609.0	1409.0 ± 759.0	1816.5 ± 16.5
LPS	10	16021.0 ± 3611.0	2271.0 ± 232.0	19084.5 ± 1112.5	667.5 ± 18.5
GRN	100	15600.5 ± 1205.5	1386.0 ± 201.0	19294.0 ± 323.0	3069.0 ± 206.0
	500	20061.5 ± 663.5	2184.0 ± 95.0	6615.0 ± 2462.0	2840.5 ± 934.5

RAW264.7 cells were stimulated with GRN 100, 500 μg/ml and LPS 10 μg/ml for 24 h at 37 °C in a humidified 5% CO_2 incubator. Three-fold diluted culture supernatants and cell lysates were utilized for thymocyte proliferation assay as described in Materials and Methods, by addition of anti-IL-1α neutralization antibody to those samples.

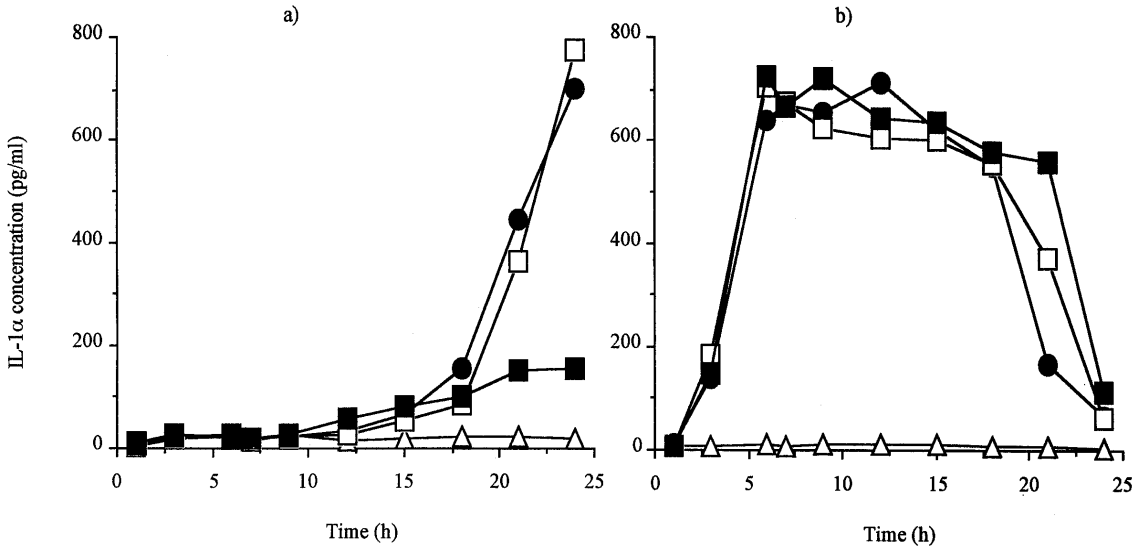

Fig. 5. Kinetics of IL-1α Production by RAW264.7 Stimulated with GRN and LPS

a) Supernatant, b) Lysate. RAW 264.7 cells were stimulated with GRN 100 μg/ml (□), 500 μg/ml (■), LPS 10 μg/ml (●) or were unstimulated (△) for 24 h at 37 °C in a humidified 5% CO_2 incubator. IL-1 α activities in the culture supernatants from RAW 264.7 cells were measured as described in Materials and Methods.

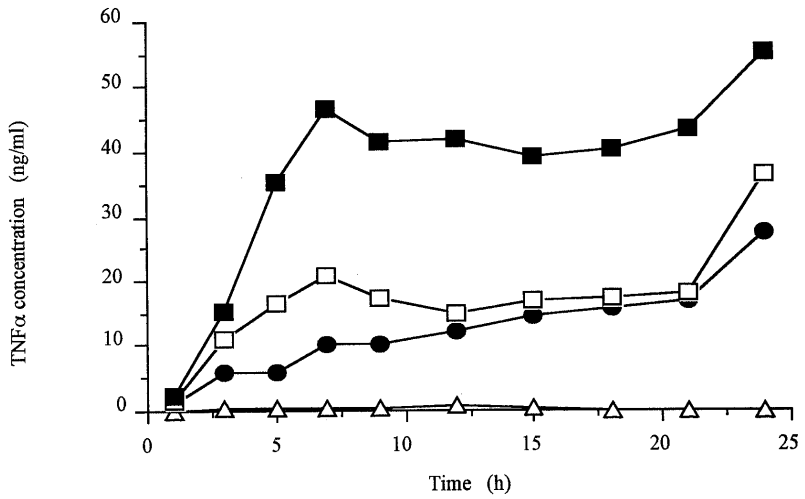

Fig. 6. Kinetics of TNF α Production by RAW 264.7 Stimulated with GRN and LPS

RAW 264.7 cells were stimulated with GRN 100 μg/ml (□), 500 μg/ml (■), LPS 10 μg/ml (●) or were unstimulated (△) for 24 h at 37 °C in a humidified 5% CO_2 incubator. TNF α activities in the culture supernatants from RAW 264.7 cells were measured as described in Materials and Methods.

RAW264.7 cells was measured by ELISA (Fig. 6). TNFα induced with 500 μg/ml of GRN was significantly higher than that induced by LPS. The amount of TNFα induced with 10 μg/ml of LPS was the same as that induced by treatment with 100 μg/ml of GRN. Thus, 500 μg/ml of GRN was most effective for TNFα production in this study. These results suggested that the activation pathway for TNFα production by GRN is substantially different from that for IL-6 and IL-1α.

Effect of Other Soluble and Gel-Forming (1→3)-β-D-Glucans on Cytokine Production by RAW264.7 Since GRN could activate macrophages to produce IL-1α, IL-6 and TNFα *in vitro*, we investigated whether other soluble and gel-forming (1→3)-β-D-glucans can induce inflammatory cytokine production by macrophages. The other gel-forming (1→3)-β-D-glucans used were SPG and SSG, and LAM and DEX were used as soluble β-glucan and α-glucan, respectively. The primary structure of SPG is the same as that of GRN (Fig. 1) although the molecular weight of single chain SPG is 150000 which is lower than that of GRN (M_r=500000).[2] SSG obtained from the liquid-culture filtrate of the fungus *Sclerotinia scleotiorum* IFO 9395 belonging to Ascomycotina consists of a β-(1→3)-polyglucose backbone with every second residue 6-*O*-substituted by mono-glucosyl branches (M_r> 5000000).[17,18] These β-glucans are known as BRM with apparent anti-tumor activity as reported elsewhere,[2,6-9,17,18] but LAM from *Laminaria digitata* has no such activity. RAW264.7 cells were cultured with 100 and 500 μg/ml of SPG at 37 °C for 24 h (Table IV), but the resultant culture supernatant did not show significant levels of cytokine production. RAW264.7 cells cultured with 100, 250 and 500 μg/ml of SSG, LAM and DEX at 37 °C for 24 h (Table V) also did not induce

TABLE IV. IL-6 and TNFα Production by RAW264.7 Stimulated with GRN and SPG

Cell culture treatment (μg/ml)		IL-6 (ng/ml)	TNFα (ng/ml)
Nil		0.260± 0.260	0.529±0.033
GRN	100	11.050± 5.580	14.458±0.156
	500	22.130±14.820	20.780±0.014
SPG	100	0.010± 0.440	0.292±0.060
	500	0.600± 0.000	0.405±0.074

RAW264.7 cells were stimulated with or without GRN and SPG (100, 500 μg/ml) for 24 h at 37 °C in a humidified 5% CO_2 incubator. IL-6 activities and TNFα production in the culture supernatants from RAW264.7 cells were measured as described in Materials and Methods.

TABLE V. Effect of GRN and the Other Soluble β-Glucans on Cytokine Production by RAW264.7

Cell culture treatment (μg/ml)	TNFα concentration (ng/ml)			
	0	100	250	500
Nil	0.000±0.000			
GRN		5.726±1.513	9.077±0.579	N.D.
SSG		0.158±0.158	0.000±0.000	0.679±0.041
LAM		0.645±0.225	0.104±0.104	1.380±0.148
DEX		0.000±0.000	0.078±0.078	0.000±0.000

RAW264.7 cells were stimulated with or without GRN (100, 250 μg/ml) and SSG, LAM, and DEX (100, 250, 500 μg/ml) for 24 h at 37 °C in a humidified 5% CO_2 incubator. TNFα production in the culture supernatants from RAW264.7 cells were measured as described in Materials and Methods.

TNFα release.

DISCUSSION

Macrophages play an important role in the augmentation of host defense systems.[1,2] For instance, it is known that macrophages activated with bacterial products including LPS and muramyl dipeptide (MDP) release several inflammatory cytokines, IL-1, IL-6, IL-8, G-CSF and TNFα, which induce immune response.[13,14] Therefore, the ability of some materials to induce cytokines from the macrophages is thought to be a parameter of their role as immunopotentiators.

Our previous studies showed that GRN is a gel-forming (1→6)-branched(1→3)-β-D-glucan and possesses host-mediated anti-tumor activity *in vivo* (Fig. 1 and Table I).[6-8,28] In this study, we initially examined the ability of GRN to induce IL-6 release from macrophages and compared the difference in the pattern of cytokine release between GRN and LPS. GRN dose-dependently stimulated RAW264.7 cells to produce IL-6, and the most effective dose tested was 500 μg/ml (Tables I and II, Fig. 2). The protein-level of IL-6 induced with GRN was quite similar to that induced with 10 μg/ml of LPS (Fig. 3). The activity observed by stimulation with GRN was not due to contamination of LPS because GRN contained negligible levels of endotoxins and could significantly induce IL-6 even using C3H/HeJ mice macrophages (Table II). Moreover, in the examination by the RT-PCR method, the ability of GRN to induce IL-6 mRNA was slightly slower than that of LPS (Fig. 4). The difference in the time-course of mRNA expression between the two stimulators suggested that GRN could stimulate the expression of IL-6 mRNA independently of LPS.

We also investigated whether GRN activated RAW-264.7 cells to produce IL-1 and TNFα. The IL-1 activities in culture supernatants and cell lysates from GRN-, LPS-treated RAW264.7 cells were stopped completely by addition of anti-IL-1α neutralization antibody (Table III). This indicated that GRN and LPS could not augment IL-1β production of RAW264.7 cells. GRN and LPS showed similar action on IL-1α production in cell lysate within a short period of time after the incubation (Fig. 5b). However, with respect to the level of IL-1α secretion in supernatant after 24 h of incubation, the IL-1 level induced with 500 μg/ml of GRN was lower than that induced with 10 μg/ml of LPS or 100 μg/ml of GRN (Fig. 5a). IL-1α was first translated as a precursor of IL-1α in cytosol, cleaved by a protease and then mature IL-1α was released from the cell membrane.[29] Therefore, 500 μg/ml of GRN might affect the processing of IL-1α on the cell membrane in activation of RAW264.7 however, further studies are required to clarify this. TNFα induction with 500 μg/ml of GRN was significantly higher than with 10μg/ml of LPS (Fig. 6) although the time course of TNFα production induced by the two was quite similar. This result demonstrated that GRN is more efficient than LPS in induction of TNFα by RAW264.7. IL-1 plays a key role in the cytokine network and is important in T cell activation in immune responses.[13,30] TNFα is a cytokine with tumor necrosis activity secreted mainly by macrophages, and was recognized as an important host regulatory molecule.[13,31] These cytokines might affect not only tumor cells but many kinds of normal cells. It was suggested that the activity of (1→3)-β-D-glucan on TNFα induction may contribute to local anti-tumor activity in tumor-bearing hosts and to various immunomodulating effects.

We further investigated whether the other soluble and gel-forming (1→3)-β-D-glucans, SPG, SSG and LAM induced cytokine production by macrophages *in vitro* in the same way as GRN. As shown in Tables IV and V, however, we confirmed that GRN is the only (1→3)-β-D-glucan able to induce cytokines from macrophages among the soluble and gel-forming (1→3)-β-D-glucans tested in this study. The primary structure of SPG is quite similar to that of GRN (Fig. 1),[2] and its molecular weight is higher.[17,18] Therefore, our results indicate that cytokine-inducing ability of soluble and gel-forming (1→3)-β-D-glucans and recognition mechanisms of (1→3)-β-D-glucans on macrophages are not restricted only by molecular weight and the ratio of branching, but also by the ultrastructure of β-glucans. This speculation is supported by previous reports which showed that lower molecular weight GRN (M_r<20000) has no ability to induce cytokine production or anti-tumor activity.[28] Recently, a certain receptor specific for (1→3)-β-D-glucans was suggested to be present on macrophages/monocytes which contributes to phagocytosis and secretion of various inflammatory mediators.[32-34] The β-glucan receptor was first demonstrated by the antagonistic effect of soluble β-glucans on phagocytosis of particulate β-glucan.[35] Abel and Czop showed that this receptor was related to

production of TNFα and IL-1 by monocytes stimulated with particulate β-glucan.[36] Furthermore, particulate β-glucans including yeast β-glucan and zymosan triggered the production of reactive oxygen species by macrophages, but not by gel-forming β-glucan such as GRN, lentinan, or SPG. Thus, the ability of GRN to activate macrophages *in vitro* is significantly different from that of particulate β-glucans. These phenomena suggest that pathways for cytokine production induced by β-glucans are not comparable to those inducing production of reactive oxygen species and may imply that the activity of particulate β-glucans is evoked through multiple recognition units on phagocytes; one of them is a receptor for soluble β-glucans and the others are specific for particulate properties. GRN is a gel-forming (1→3)-β-D-glucan constructed by a definitive helical and randomly coiled glucosyl chain.[3,4] The unique activity of GRN in cytokine production might result from the complexity of its ultrastructure.

Since GRN has been reported to exhibit various immunomodulating activities, we focused on its activation of macrophages, and investigated its capacity to augment production of cytokines by macrophages. The present study demonstrated that GRN induced IL-6 production from the murine macrophage cell line and from murine peritoneal macrophages *in vitro*. Moreover, it induced the other inflammatory cytokines such as IL-1α and TNFα. IL-6 plays a crucial role in host immune response, acute protein synthesis and the maintenance of homeostasis. It can be speculated that IL-6 induced by administration of GRN has effects on several biological activities as in the case of IL-1 and TNFα.[37] We also found that the pattern of cytokine production induced with GRN was different from that with LPS. The above data demonstrates that GRN is a novel macrophage activator *in vitro*. Further investigations to clarify its activation mechanisms, including the discovery of the specific receptor for (1→3)-β-D-glucans and the signaling pathway for its activation, remain to be performed.

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