Activation of Murine Kupffer Cells by Administration with Gel-Forming (1→3)- β -D-Glucan from *Grifola frondosa*

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The effect of gel-forming (1→3)- β -D-glucan on the immunological activities of murine kupffer cells was examined. A branched type gel-forming (1→3)- β -D-glucan, GRN, was administered intravenously to mice. GRN associating to kupffer cells was detected by an immunohistochemical technique using anti-GRN antibody. A kinetic study of the activation of kupffer cells revealed that GRN could induce the enhanced production of cytokines and nitric oxide on 4 to 7 d after the administration. The activities are further augmented by adding GRN in the culture. The cytostatic activity of kupffer cells against murine lymphoma, EL-4, was also augmented by a time course similar to nitric oxide production. The cytostatic activity was reduced by adding an inhibitor of nitric oxide synthase, implying that the cytostatic activity of kupffer cells to EL-4 was dependent on nitric oxide. The administration of GRN increased the expression of CD11b, known as a β -glucan receptor, on kupffer cells at day 7. The above data suggest that GRN could activate murine kupffer cells to enhance the production of cytokines and nitric oxide, and that the activation required 4 or 7 d, at least, after the administration with GRN.

Key words grifolan (GRN); kupffer cell; cytostatic activity; nitric oxide; cytokine; CD11b

Grifolan (GRN) is a gel-forming (1→3)- β -D-glucan from *Grifola frondosa* and is known to be an immunopotentiator with host-mediated antitumor activities.¹⁾ One of the most important activation mechanisms of β -glucans on the host defense system is the enhancement of macrophage function.²⁾ Macrophages are known as important cells for providing initial host defense signals by producing various cytokines and cytotoxic effects on tumor cells.^{3,4)} In the case of peritoneal macrophages (PM), GRN has shown an ability to induce some inflammatory cytokines, such as tumor necrosis factor- α (TNF- α), interleukin-1 α (IL-1 α), and interleukin-6 (IL-6).⁵⁾ Furthermore, the cytostatic activity of PM against murine tumor cells was enhanced by GRN-administration.^{6,7)} It has been clarified that most β -glucans are distributed to the reticuloendothelial system, such as the liver and spleen, in mouse after systemic administration.^{8,9)} Suda *et al.* reported that kupffer cells play an important role in the metabolic degradation of a soluble β -glucan.¹⁰⁾ However, little is known concerning the effect of GRN on the function of kupffer cells. Therefore, we have focused on the examination of kupffer cells' activity induced by administration of GRN. To examine several activities of kupffer cells, the production of cytokines and nitric oxide (NO) and the cytostatic activity of kupffer cells were tested. This paper will report the activation of several functions of kupffer cells by GRN administration.

MATERIALS AND METHODS

Mouse Male ICR mice (6 to 10 weeks of age) were purchased from Japan S.L.C. (Hamamatsu, Japan) and placed under specific pathogen free conditions.

Preparation of GRN GRN was prepared from a liquid culture of the mycelium of *Grifola frondosa* as described in other reports.¹⁾ Contamination of endotoxin in the GRN preparation was less than 0.0001%, as assessed by Endospecy (Seikagaku Kogyo, Japan). It has been reported that several effects of GRN on peritoneal macrophages were not due to the trace level of endotoxin in the GRN preparation.⁵⁾

Administration of GRN GRN (250 μ g/0.1 ml) dis-

solved in pyrogen-free saline (Otsuka, Japan) was injected intravenously to ICR mice once on days 7, 4, and 1, respectively. The control mice were injected with pyrogen-free saline (Otsuka, Japan) alone under the same condition as GRN-treated mice.

Isolation of Kupffer Cells Cell preparation was performed as described in an earlier report, with slight modification.¹¹⁾ The mice were sacrificed on day 1, 4 and 7 after the administration of GRN. The liver was perfused with 25 ml of phosphate buffered saline (PBS, pH 7.2) to remove peripheral blood cells. The liver was excised and minced with scissors into 1- to 2-mm³ fragments. The fragments were digested for 3 h at room temperature in a triple enzyme solution containing 0.4% collagenase (w/v), 0.02% deoxyribonuclease (DNase, w/v), 30 U/ml hyaluronidase, and 10% fetal calf serum (FCS) in RPMI 1640 medium (RPMI-FCS) in an orbital shaker. The liver digest was then washed twice with RPMI-FCS. The liver cells were resuspended in RPMI-FCS and placed on Histopaque-1077 (Sigma) in centrifuge tubes. The tubes were centrifuged at 700 $\times g$ for 30 min at room temperature. The cell layer between RPMI-FCS and Histopaque-1077 was carefully removed and washed twice with RPMI-FCS. The cells were layered on a 90 mm culture dish (Sumilon), and incubated at 37 °C for 15 min in a humidified 5% CO₂ incubator. After the incubation, non-adherent cells were aspirated, and the dish was gently washed with RPMI-FCS. The remaining adherent cells were scraped from the plate, washed with RPMI-FCS, and counted by trypan blue dye exclusion test. The morphology of the cells was assessed by Diff Quick staining. To determine cell purity, cells were incubated with fluoresceinisothiocyanate (FITC)-labeled latex beads (diameter 1.87 μ m, Polyscience, Inc., Warrington, PA) for 1 h at 37 °C. The percentage of cells which phagocytosed the latex beads was 78.4%.

Immunohistochemical Study of Kupffer Cells by Staining with Anti-GRN Antibody Kupffer cells were placed on a glass slide by Cytospin, and fixed with 1% paraformaldehyde for 30 min at 4 °C. The cells were incubated with PBS containing 10% goat serum for 30 min at

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room temperature, then further incubated with anti-GRN antibody overnight at 4°C. The cells were then washed with ice-cold PBS, treated with methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase, and incubated with anti-rabbit IgG conjugated with horseradish peroxidase. The localization of GRN associating kupffer cells was revealed by incubation with 3,3'-diaminobenzidine as brown dots.

Cell Culture for Cytokine Production Kupffer cells (2×10^6 cells/ml) isolated from the mice were suspended in RPMI-FCS and cultured in a 12 well plate (Sumilon) for 24 h at 37°C in a 5% CO₂ incubator. Supernatants of the culture were collected and the remaining cells on each well were lysed in distilled water by repeated freeze and thaw as described elsewhere.¹²⁾ The lysates were filtrated through cellulose acetate filters (pore size 0.2 µm).

ELISA for Cytokines Quantification of the cytokines in the culture supernatant and lysate were performed by a specific sandwich ELISA system as described in other reports.⁵⁾ Microtiter ELISA plates (Sumilon) were coated with the capture antibody for IL-1α (hamster IgG; Genzyme) or IL-6 (rat-IgG1; Pharmingen). The antibody was diluted in bicarbonate buffer (pH 9.6) to a final concentration of 1.5 µg/well, and 50 µl were added to each well. Plates were incubated at 4°C overnight, followed by washing three times with PBS containing 0.05% Tween 20 (PBS-T). Blocking was achieved by adding 100 µl/well of PBS-T containing 0.5% BSA (BPBS-T) and then incubating the mixture at 37°C for 40 min. After three more wash cycles, the rmIL-1α (Genzyme) or rmIL-6 (R and D Systems) and supernatant samples were added to the plate (50 µl/well) and left to incubate for 40 min at 37°C. After three washings with PBS-T and blocking with BPBS-T for 10 min, the bound soluble cytokine was detected using an anti-mIL-1α polyclonal antibody (Genzyme) or biotin labeled anti-mIL-6 monoclonal antibody (mAb) (Pharmingen). Antibodies were diluted in BPBS-T, and 50 µl/well were added for 40 min at 37°C. The plates were washed another three times, and 50 µl of a 1:10000 diluted anti-rabbit IgG-horseradish peroxidase (Cappel™) (avidin-horseradish peroxidase for IL-6) was added to each well. After incubation for 40 min and three final washes with PBS-T, the plates were developed using 50 µl/well of tetramethylbenzidine (Kirkegaard and Perry Laboratories, Inc.). The reaction was stopped by the addition of 50 µl 1 N H₃PO₄/well and read using a microplate reader (MTP-32, Corona Electric Co., Ltd.) at 450 nm.

Assay for NO After incubating the kupffer cells (2.5×10^5 cells in 100 µl RPMI-FCS) for 24 h, the synthesis and release of NO by the kupffer cells were determined by assaying the culture supernatant for nitrite content. Briefly, 100 µl supernatant was reacted for 10 min at room temperature with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diaminedihydrochloride, 2.5% phosphoric acid).¹³⁾ The optical density was measured at 550 nm (Ref. 630 nm). The nitrite content was quantified by comparison with a standard curve generated with sodium nitrite in the range of 0 to 100 µM. To examine the contribution of nitric oxide synthase (NOS) for the phenomenon, an L-arginine analog, N-monomethyl L-arginine (NMMA), was added to the culture at the concentration of 25 µg/ml.

Cytostatic Activity After an i.v. injection of GRN

(250 µg/mouse), the kupffer cells were collected. One hundred µl of suspension (5×10^6 cells/ml) in phenol red free RPMI (Sigma) containing 10% FCS was placed in a flat-bottomed 96-well culture plate. Thereafter, 100 µl of lymphoma EL-4 cell suspension (5×10^5 or 1×10^6 cells/ml) in phenol red free RPMI-FCS was placed in each well, followed by culturing for 48 h. Four hours before the end of culture, 10 µl of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1, Dojin) dissolved in 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS, Dojin) at 3.26 mg/ml was added to each well. The optical density of the culture medium was measured at 450 nm (Ref. 630 nm). The cytostatic activity was calculated using the following formula:

$$\text{cytostatic activity (\%)} = [1 - (\text{OD}_{450} \text{ of kupffer cells} + \text{EL-4}) / \{(\text{OD}_{450} \text{ of kupffer cells}) + (\text{OD}_{450} \text{ of EL-4})\}] \times 100$$

Flow Cytometry Cells were adjusted to a concentration of 5×10^5 cells/0.1 ml in PBS containing 0.1% sodium azide and 1% FCS. For direct single color immunofluorescent staining, the cells were incubated with FITC-labeled mAb at 4°C for 30 min. The mAbs (M1/70 for CD11b and N418 for CD11c) were prepared from a serum-free culture supernatant of the hybridoma obtained from American Type Culture Collection. The stained cells were washed two times with PBS-azide buffer, then resuspended in 1% paraformaldehyde. The cells were examined by single-color flow cytometry using FCS-1 (Japan Spectroscopic Co.).

RESULTS

Association of GRN to Kupffer Cells Kupffer cells were isolated from the mice on days 7, 4 and 1 from the time

Fig. 1. Immunohistochemical Staining of Kupffer Cells Using Anti-GRN Antibody

Kupffer cells were placed on a glass slide by cytospin, and were stained with anti-GRN polyclonal antibody (rabbit) and horse radish peroxidase-conjugated anti-rabbit IgG (goat) after blocking with 10% goat serum. GRN in cells were visualized by reacting diaminobenzidine as brown dots, and cells were counter-stained with methyl green. magnification: $\times 40$.

Fig. 2. IL-1 α Production by Kupffer Cells Obtained from GRN-Administered Mice

Kupffer cells isolated on days 0, 1, 4, and 7 after administration of GRN were cultured for 24 h at 37°C in the presence of 250 μ g/ml GRN or 1 μ g/ml LPS. Each culture supernatant (a) and cell lysate (b) was prepared as described in Materials and Methods. IL-1 α content was determined using an ELISA. Statistical significance against day 0 was determined by Student's method * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Six mice in each group were used to obtain data. Error bars represent standard deviation (S.D.).

of administration of GRN. Kupffer cells were placed on a glass slide by cytopspin, then stained with anti-GRN antibody and subsequently with methyl green.¹⁴⁾ The cells were positively stained with the antibody as indicated by brown dots of peroxidase products (Fig. 1). However, non-adherent cells from the liver did not show such results (data not shown). These data indicated that kupffer cells could associate to GRN after i.v. administration.

Effect of GRN on Cytokine Productivity of Kupffer Cells The production of inflammatory cytokines (IL-1 α and IL-6) by kupffer cells was examined after the administration with GRN. Kupffer cells were incubated in RPMI-FCS with GRN 250 μ g/ml or lipopolysaccharide (LPS) 1 μ g/ml at 37°C for 24 h. Cytokines in the culture supernatant and cell lysate were measured by ELISA. As shown in Fig. 2a, IL-1 α production in the culture supernatant was induced in kupffer cells. The activity was increased in accordance with the time after administration. There was no significant change in IL-1 α level in the supernatant from the culture of kupffer cells with GRN or LPS. However, the IL-1 α in the cell lysate was increased by adding GRN and LPS (Fig. 2b). Especially, kupffer cells from the mice on days 4 to 7 after GRN administration showed higher levels of IL-1 α by stimulation with

Fig. 3. IL-6 Production by Kupffer Cells Obtained from GRN-Administered Mice

Kupffer cells isolated on days 0, 1, 4, and 7 after administration of GRN were cultured for 24 h at 37°C in the presence of 250 μ g/ml GRN or 1 μ g/ml LPS. The IL-6 content in each culture supernatant was determined using ELISA as described in Materials and Methods. Statistical significance against day 0 was determined by Student's method * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Six mice in each group were used to obtain data. Error bars represent S.D.

Fig. 4. NO Production by Kupffer Cells Obtained from GRN-Administered Mice

Kupffer cells isolated on days 0, 1, 4, and 7 after administration of GRN were cultured for 24 h at 37°C. NO content in the culture supernatant was determined using Griess reagent as described in Materials and Methods. Statistical significance against day 0 was determined by Student's method * $p < 0.01$. Six mice in each group were used to obtain data. Error bars represent S.D.

GRN and LPS.

In the case of IL-6 production, GRN and LPS stimulated kupffer cells on days 4 and 7 after the administration with GRN (Fig. 3). However, kupffer cells could not produce a higher level of IL-6 when the cells were incubated without GRN or LPS. These results suggest that kupffer cells are activated by the administration of GRN, which is then responsible to GRN or LPS for producing inflammatory cytokines.

NO Production by Kupffer Cells NO production by kupffer cells was investigated. After the i.v. administration of GRN, kupffer cells were isolated and cultured in RPMI-FCS for 48 h at 37°C. The NO content in the culture supernatant was assessed with Griess reagent. NO production by the kupffer cells on day 0 or 1 after the administration of GRN was around 3 to 5 μ M, but an incremental increase in NO production was observed in the kupffer cells on days 4 and 7 (Fig. 4). On day 7, the NO production was 6-fold higher than on days 0 or 1. These results are comparable to the cytokine

Fig. 5 Cytostatic Activity of Kupffer Cells against EL-4 Lymphoma

Kupffer cells (5×10^5 cells) isolated on days 0, 1, 4, and 7 after administration of GRN were cultured with EL-4 cells (1×10^5 cells or 5×10^4 cells) for 44h at 37°C in a 96-well culture plate. To assess the proliferation of EL-4 cells, WST-1 was added to the culture plate and further incubated for 4h at 37°C . The absorbance at 450 nm of culture supernatant was measured and cytostatic activity of kupffer cells in triplicate culture was calculated as described in Materials and Methods. Significance to control (day 0) in corresponding effector/target (E/T) ratio: * $p < 0.05$. Error bars represent S.D.

Fig. 6 Effect of NOS Inhibitor on Cytostatic Activity of Kupffer Cells

Kupffer cells on day 7 after GRN- or saline-administration were cultured with EL-4 as described in Fig. 5 at an effector/target ratio=10/1 in the presence of saline or $25 \mu\text{g/ml}$ of NMMA. Cytostatic activity was calculated as described in Materials and Methods. Significance to saline-treated group: * $p < 0.01$. Error bars represent S.D.

production of kupffer cells described above.

Cytostatic Activity of Kupffer Cells Activated with GRN To examine the effect of the administration of GRN on the cytostatic activity of kupffer cells, the cells were isolated and cultured with tumor cells, EL-4, for 48h at 37°C . The proliferation of EL-4 was assessed by a modified 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) method as described by Scudiero *et al.*¹⁵⁾ Kupffer cells from mice on days 4 and 7 showed a higher inhibitory effect on the proliferation of EL-4 (Fig. 5). These results also coincided with the influence of GRN on the production of cytokines and NO by kupffer cells.

Effect of NOS Inhibitor on Cytostatic Activity and NO Production of Kupffer Cells In order to examine how the cytostatic activity of kupffer cells is induced, we investigated the effect of NO on the cytostatic activity against EL-4. A culture which included kupffer cells and EL-4 was performed in the presence and absence of NMMA. After 48h incubation, the augmentation of cytostatic activity by GRN was significantly reduced to the control level by adding NMMA (Fig. 6). The NO synthesis was also monitored by Griess

Fig. 7 NO Production by EL-4 and Kupffer Cells Obtained from GRN-Administered Mice

Kupffer cells isolated on days 0 (■) and 7 (□) after the administration of GRN were cultured for 48h at 37°C with or without EL-4 lymphoma and $25 \mu\text{g/ml}$ NMMA as indicated. NO content in the culture supernatant was determined using Griess reagent as described in Materials and Methods. Statistical significance to the absence of NMMA was determined by Student's method * $p < 0.001$. Error bars represent S.D.

Fig. 8 Flow Cytometry of Kupffer Cells Obtained from GRN-Administered Mice

Kupffer cells isolated on days 1 and 7 after the administration of GRN were treated with FITC-conjugated anti-CR3 or CR4 mAb. A histogram of cell fluorescence was composed of 10000 cells in each panel.

reagent. As shown in Fig. 7, the level of NO in the culture supernatant was almost diminished with NMMA. These data strongly suggest that the cytostatic activity augmented by GRN was induced by NO from kupffer cells.

Phenotypic Change of Kupffer Cells by Administration of GRN Kupffer cells were further examined by flow cytometry to measure the expression of receptors on the cell surface. Kupffer cells were immunochemically stained with FITC-conjugated mAb for CD11b (CR3) and CD11c (CR4). As shown in Fig. 8, the expression of CR4, which has recently been reported as one of functional LPS receptors,¹⁶⁾ remained unchanged even after administration of GRN on any day. However, the expression of CR3 on kupffer cells was increased in the case of GRN on day 7. This result suggests that not only the production of cytokines and NO, but also receptor expression, is enhanced by the administration of GRN.

DISCUSSION

In this study, we have shown that (1 $\rightarrow$ 3)- β -D-glucan from *Grifola frondosa* enhanced several immunological activities of kupffer cells. Kupffer cells appeared immunohistochemically positive by staining with anti-GRN antibody, suggesting that GRN can associate with kupffer cells by i.v. administration. Using radio isotope-labeled β -glucan, grifolan NMF-5N and *Sclerotinia sclerotiorum* glucan (SSG) which is obtained from *Sclerotinia sclerotiorum* IFO9395, it was demonstrated that those mainly accumulated in the liver.^{8,9)} Suda *et al.* demonstrated that most of the SSG injected to mice is not incorporated into liver cells, but is associated with matrix proteins which are susceptible to collagenase digestion.⁹⁾ However, a small amount of glucans remain in the cellular portion in the liver.⁹⁾ This study demonstrated that β -glucan can associate with non-parenchymal cells, but not parenchymal liver cells, even after collagenase digestion. Since GRN was also detected in the supernatant of a collagenase-digested liver cell suspension by ELISA specific to GRN, GRN is likely to bind to a non-cellular substance as a matrix protein (data not shown).

It has been reported that GRN can enhance the cytokine production of peritoneal macrophages or the RAW264.7 macrophage cell line *in vitro*.⁵⁾ The cytokine production of macrophages stimulated by GRN was not dependent on trace levels of endotoxin, since peritoneal macrophages from an LPS-unresponsive mouse strain, C3H/HeJ, also produced a significant level of cytokines by stimulation with GRN.¹⁷⁾ According to the previous results, we further tested the ability of kupffer cells to produce inflammatory cytokines after the administration of GRN. Kupffer cells from GRN-administered mice showed increased production of IL-1 α and IL-6. The production of IL-1 α by stimulation with GRN or LPS *in vitro* showed a significant difference between culture supernatants and cell lysates. (Figs. 2a and b). It was reported that IL-1 production in the supernatants of LPS-treated kupffer cell cultures was quite low in the case of a non-toxic concentration of LPS (100 ng/ml not 10 μ g/ml).¹⁸⁾ Our findings were comparable to those reports, although we used 1 μ g/ml LPS from *Escherichia coli* O111. The maturation of IL-1 protein, which was induced by a certain stimulus, might not be activated in kupffer cells.¹⁸⁾ The production of TNF- α from the kupffer cells was less than the detectable level of this ELISA system. It has been reported that kupffer cells produce lower amounts of TNF- α with several stimulators other than peritoneal macrophages.¹⁹⁾ Our data appear to be comparable to those findings on the TNF- α production. Nevertheless, the low productivity of TNF- α , cytostatic activity of kupffer cells against EL-4 lymphoma was significantly augmented by the administration of GRN. Other effector molecules for exhibiting tumoricidal effects were tested. NO has been investigated concerning its physiological role. In a host defense, NO is believed to be important for degrading microorganisms or some tumor cells.¹³⁾ As shown in Fig. 4, an elevated level of NO was observed in the culture supernatant of kupffer cells isolated on days 4 and 7 after the administration of GRN. A similar time course of activation of cytostatic activity and NO production led to a hypothesis that NO is one factor inhibiting tumor cell growth. To confirm this, an inhibitor of NO synthesis (NMMA) was added to the culture of the cy-

tostatic test. The cytostatic activity of kupffer cells from GRN-administered mice was reduced almost to the control level, suggesting that NO acted mainly as an effector molecule on the cytostatic activity induced by GRN. The present paper is the first, to our knowledge, to demonstrate that β -glucan can enhance the cytostatic activity of kupffer cells by augmenting NO synthesis.

CR3 has been reported to be a β -glucan receptor of phagocytes.²⁰⁾ GRN can also be recognized by CR3 on human neutrophils.²¹⁾ Furthermore, Zarewycz *et al.* reported that CR3 acts as a co-receptor for the LPS-LPS binding protein complex on human neutrophils.²²⁾ A recent report demonstrated that CR3 can transmit signals to intracellular portion by treatment with LPS.²³⁾ Therefore, we examined the expression level of CR3 on kupffer cells. Kupffer cells obtained from the mice on day 7, but not day 1, showed an elevated level of CR3 expression, suggesting an indirect effect of GRN on the increased expression of CR3. These cellular events, including changes in phenotype and cellular function, might be regulated by other cell populations, *i.e.* lymphocytes. In other reports, the effect of an elevated level of CR3 on rat kupffer cells has been shown using human recombinant IL-2 for kupffer cell activation.²⁴⁾ Our group also reported that the administration of β -glucans enhanced the production of interferon- γ (IFN- γ) by which macrophages are activated.²⁵⁾ Therefore, we speculated that CR3 expression resulted from an amplification of the cytokine network involving kupffer cells and lymphocytes which were initiated with GRN. This speculation helps explain why the enhanced activities of kupffer cells such as cytokine production and NO synthesis required 4 or 7 d after GRN-administration to occur. Although there is no direct evidence to suggest the contribution of lymphokines in activating kupffer cells by administration with GRN, the present paper provides one mechanism of activation of the host immune system by β -glucans.

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