Analysis of Cytokine mRNAs Induced by the Administration of a Highly Branched (1→3)- β -D-Glucan, OL-2

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OL-2, a highly branched (1→3)- β -D-glucan, is an antitumor glucan showing strong hematopoietic activity with weaker adjuvant activity than schizophyllan (SPG), another antitumor glucan and one which is used clinically. This paper deals with the gene expression of cytokines in mice by OL-2 and SPG in order to characterize their immunopharmacological activity. Gene expression was examined by a reverse transcriptase–polymerase chain reaction method after intraperitoneal administration of OL-2 or SPG (250 μ g/mouse). The OL-2 administered mice strongly expressed the interleukin 1 receptor antagonist (IL-1ra) gene but SPG administered mice did not. The difference would be strongly related to the antigen-specific response between OL-2 and SPG. In the genes related to haematopoiesis, OL-2 induced G-CSF and GM-CSF, but SPG induced IL-3. These differences would relate to the pattern of haematopoietic response. Comparing the cytokine gene expression in ICR and AKR mice by OL-2 administration, the changes in cytokine gene expression were less in AKR mice administered OL-2. These findings suggest that the immunopharmacological characteristics of OL-2 are closely related, at least in part, to the activation of the complement system. The data shown in this paper also suggest that cytokine gene expression by β -glucan would be significantly affected by the structure of these glucans.

Keywords Leiwan (*Omphalia lapidescens*); OL-2; β -glucan; polymerase chain reaction; cytokine

Lentinan (LNT)¹⁾ and schizophyllan (SPG)²⁾ are (1→3)- β -D-glucans now being used clinically in Japan as immunopotentiators for cancer therapy. The mechanism of the antitumor activity of (1→3)- β -D-glucan has been demonstrated to be the activation of the host immune system³⁾ through the regulation of cytokines in the cytokine network and activation of the complement system,⁴⁾ and considerable experimental evidence supports this finding. We have studied the antitumor activities and the structure–activity relationships of (1→6)-branched (1→3)- β -D-glucans, GRN (one branch in every third main chain unit; 1/3), SSG (1/2) and OL-2 (1/3). However, there exists only limited knowledge of early biochemical events leading from the initial recognition of the glucan molecules by the host, such as activation of the complement⁴⁾ and blood coagulation systems.⁵⁾ Furthermore, although a certain glucan-specific receptor protein was suggested to be presented on phagocytic cells,⁶⁾ this is not enough to fully explain the mechanism of the antitumor activity. To elucidate this mechanism(s) of the biological activities of these glucans, it is necessary to understand the above structure–activity relationship at the molecular level.

To address the above question, we have started the analysis of cytokine regulation by using a reverse-transcriptase assisted polymerase chain reaction (RT-PCR). We have already found that gene expression of the interleukin 1 family, IL-1 α , IL-1 β , and IL-1 receptor antagonist (IL-1ra), were different between SPG and OL-2.⁷⁾ In addition to IL-1, dozens of cytokines were identified in human as well as murine. These cytokines were believed to systematically regulate the immune system in an orchestrated manner.⁸⁾ These cytokines would contribute to, at least in part, the immunopharmacological activity of (1→3)- β -D-glucans.

A highly branched (1→3)- β -D-glucan, OL-2, extracted from *Omphalia lapidescens* showed antitumor activity on

the solid form of sarcoma 180 when administered intralesionally (i.l.), and increased the life span of mice with ascites form of sarcoma 180 and MH134 hepatoma.^{9,10)} In addition, OL-2 showed significant activity on the alternative complement pathway and a strong induction of leukocytes in many organs.¹¹⁾ At least part of the immunopharmacological activity of OL-2 was significantly reduced in AKR mice, a murine strain genetically C5 deficient. In contrast to other (1→3)- β -D-glucans, however, OL-2 showed almost no antitumor activity against the solid form of sarcoma 180 by intraperitoneal administration and almost no adjuvant activity to an anti-sheep red blood cells (SRBC) antibody responses.¹²⁾ In addition, as described above, OL-2 showed strong expression of the IL-1ra gene.⁷⁾

These facts suggested that the structure–activity relationship of (1→3)- β -D-glucan in mediating immunopharmacological activities differs depending on the activity. Precise examination of physicochemical characteristics and immunopharmacological activities and a comparison of the data with those of antitumor glucans would help in the molecular understanding of glucan-mediated immunopharmacological activity, including the antitumor activity, and would reveal the critical role of the side chain. In this paper, we examine the expression of cytokine mRNA by the RT-PCR method¹³⁾ and discuss the immunopharmacological activities of OL-2.

MATERIALS AND METHODS

Mice Male ICR and AKR mice were obtained from Japan SLC, Inc., Shizuoka. Mice used were 5–9 weeks of age and were kept under specific pathogen-free conditions.

Materials OL-2 was prepared from *Omphalia lapidescens*^{14,15)} and contained a negligible concentration of lipopolysaccharide (LPS) assessed by a limulus test.¹⁶⁾

SPG was generously provided by Kaken Pharmaceutical Co., Ltd.

Mice were intraperitoneally administered 250 μ g of OL-2 or SPG. At appropriate periods after the administration, the mice were anesthetized and their peritoneal exudate cells (PEC), spleen and liver were removed and immediately stored at -80°C until RNA extraction.

RNA Extraction Total cellular RNA was extracted from the entire PEC and from approximately 30 mm³ each of the spleen and liver by the acid guanidium thiocyanate/phenol/chloroform extraction method.¹⁷⁾ The tissues were homogenized in 300 μ l of lysis solution (4 M guanidine, 2% Sarkosyl, 1% 2-mercaptoethanol, 10 mM EDTA), and heated to 60°C on a hot plate. To digest DNA and remove proteins, 150 μ l of RNA extraction buffer (0.1 M sodium acetate pH 5.2, 10 mM Tris-HCl pH 7.4, 1 mM EDTA), 300 μ l of Tris-saturated phenol and 300 μ l of chloroform were added to the lysates, and the mixture was thoroughly vortexed every 1–2 min during a 10 min period at 60°C . The mixture was then chilled on ice for 10 min and centrifuged at $3000 \times g$ for 10 min at 4°C . The aqueous phase was reextracted with an equal volume of phenol:chloroform (1:1) at room temperature, and the RNA was precipitated with 2 volumes of ice-cold ethanol and 0.2 volume of 3 M sodium acetate (pH 5.2) at -80°C for 2 h. The precipitates were centrifuged at $12000 \times g$ for 30 min at 4°C , the supernatant discarded, resuspended in 300 μ l of TNES (0.1 M Tris-HCl pH 7.4, 50 mM NaCl, 10 mM EDTA, 0.2% SDS, 200 μ g/ml proteinase K), and incubated for 1 h at 37°C . The mixture was heated at 60°C , then 150 μ l of extracting buffer, 150 μ l of Tris-saturated phenol and 150 μ l of chloroform at 60°C were added. The mixture was then chilled on ice for 10 min and centrifuged at $3000 \times g$ for 10 min at 4°C . The aqueous phase was recovered, and the RNA was precipitated with 2 volumes of ethanol and 0.2 volume of 3 M sodium acetate (pH 5.2) at -80°C for 2 h. The precipitates were centrifuged at $12000 \times g$ for 10 min at 4°C , the supernatant discarded, and the remainder washed once with 75% ethanol. Nucleic acid pellets were dried by a centrifugal concentrator (Tomy, Tokyo), then resuspended in 20 μ l of DEPC-treated water and stored at -80°C until subjected to cDNA preparation.

cDNA Preparation 7.5 μ l of RNA was mixed with a master mix containing 50 mM (final concentration) Tris-HCl, 75 mM KCl, 10 mM DTT, 3 mM MgCl₂, 2.5 μ M random hexamers, 1 mM dATP, dCTP, dGTP, and dTTP, 60 units of RNase inhibitor, 600 units of Moloney-Murine leukemia virus reverse transcriptase (MMLV RTase) (United States Biochemical Corporation) in a final volume of 150 μ l, and incubated at 37°C for 60 min, 99°C for 5 min, and 5°C for 5 min. Samples were stored at -80°C until subjected to PCR amplification.

PCR Procedure Aliquots of the cDNA prepared as described above 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 a 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 gels were then examined on a UV light box and photographed. Two hundred fifty ng of pHY marker (Takara) was run in parallel as a molecular weight marker (providing bands at 4870, 2016, 1360, 1107, 926, 658, 489, 267, and 80 bp). Specificities of the amplified bands were validated by their predicted sizes, by the fragmentation patterns of the digest with restriction enzyme (*Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I, *Rsa*I, and *Xho*I), and by homology to the sequences reported previously.

The primers used for this experiment were as follows:

β -actin	sense primer (s) 5'-GCCATGGATGACGATATCGCT-3'
	antisense primer (a) 5'-TCATGAGGTAGTCTGTCAGGT-3'
IL-1 α	(s) 5'-CTCTGAATCAGAAATCCTTC-3' (2) 5'-TAGGCTACATGTCAAATTT-3'
IL-1 β	(s) 5'-AAAAGATGAAGGGCTGCTTCCA-3' (a) 5'-CATGGACAATATCACTTGTGG-3'
IL-1ra	(s) 5'-CCCTGCAAGATGCAAGCCTTCA-3' (a) 5'-TTGGTCTTCTGGAAGTAGAAC-3'
IL-2	(s) 5'-AACAGCGCACCCACTTCAA-3' (a) 5'-TTGAGATGATGCTTTGACA-3'
IL-3	(s) 5'-CACCGTTTAACCAGAACGTTGA-3' (a) 5'-ATCCAGATCTCGAATGAAGACC-3'
IL-6	(s) 5'-TTCCTCTCTGCAAGAGACT-3' (a) 5'-TGTATCTCTCTGAAGGACT-3'
M-CSF	(s) 5'-AGGAACAGCTGGATGATCCTGT-3' (a) 5'-TTCGGACACAGGCCTTGTCTG-3'
G-CSF	(s) 5'-GCTTCAGCTGGATGTTGCCAA-3' (a) 5'-TCTGCTCAGGTCTAGGCCAAGT-3'
GM-CSF	(s) 5'-TTCCTGGGCATTGTGGTCTAC-3' (a) 5'-TGGATTCAGAGCTGGCCTGG-3'
TNF α	(s) 5'-CAAAGGGATGAGAAGTTCCCAA-3' (a) 5'-CTCCTGGTATGAGATAGCAA-3'

In order to compare the gene expression in each organ, at least three experiments ($n=6$) were performed, and the figures show one of the representative data. The RT-PCR reactions of each gene and OL-2 and SPG were performed separately. In less expressed genes, increasing concentrations of cDNA were used or the cycles of PCR reaction were increased.

RESULTS

Gene Expression of Cytokines mRNA by Glucan Treatments in ICR Mice The immunopharmacological activities of an antitumor glucan, OL-2, differed from those of SPG as stated in the introduction. The mechanisms of immunopharmacological activity and the difference between OL-2 and SPG were studied by examining the gene expression of cytokine mRNAs in representative organs, the peritoneal cavity, spleen, and liver, after glucan administration. A solution of OL-2 or SPG (250 μ g of each/mouse) was administered intraperitoneally. After appropriate intervals, the PEC, spleen, and liver were collected and their total RNA was prepared by the guanidium hot phenol method. The gene expression of cytokine mRNA was compared by RT-PCR using appropriate primers, as

shown in Materials and Methods. After the RT reaction of each RNA, the concentration of the resulting cDNA was adjusted by the fluorescence intensity of the PCR products of β -actin. In order to normalize the cDNA concentration, the PCR reaction of β -actin was performed using different concentrations of cDNA at least twice for all cDNA preparations. The PCR reactions of cytokines were performed by using the resulting concentration of cDNA. Experiments for the PEC, spleen, and liver, and for OL-2 and SPG, were separately examined because only a limited number of samples could be handled, so results among the PEC, spleen, and liver, and between OL-2 and SPG could not be directly compared.

Figure 1 shows a representative result of β -actin and the cytokine mRNA gene expressions of PEC obtained from the glucan administered mice. In comparison with the mRNA concentration of the control mice, OL-2-administered and SPG-administered groups revealed several interesting phenomena. In the case of the PEC obtained from OL-2 administered mice, the mRNAs of IL-1 α , IL-1 β , IL-6, TNF α , and GM-CSF were increased from 1 h and remained high for a couple of days. The mRNA of IL-2 was increased from 24 h and remained high for a couple of days. The mRNA of IL-1 β was increased from 1 h and remained high for a week. The mRNA of GM-CSF

showed two peak phases, at around 1 h and after a couple of days. The mRNA of M-CSF was slightly increased at around a week.

In the case of the PEC obtained from SPG-administered mice, the mRNAs of IL-1 α and TNF α were increased from 1 h and remained high for a week. The mRNA of IL-2 was increased at 1 h and remained high for a day. The mRNA of IL-1 β showed two peaks. Of interest, the mRNA of IL-3 was increased at a day, while the mRNAs of IL-1 α , M-CSF, and IL-6 were once decreased.

These results suggested that the pattern of mRNA expression of cytokine genes was different between OL-2- and SPG-administered mice. In comparison with the fluorescence intensity of the PCR product in the control mice of the experiments using OL-2 and SPG, unfortunately, patterns were not consistent (IL-6, IL-1 α). The control mice must show an identical cytokine profile; thus, the above difference might be related to the concentration of cDNA used and the PCR reaction cycles.

Figure 2 shows a representative result of β -actin and cytokine mRNA gene expressions from the spleen obtained from the glucan-administered mice. In the case of OL-2-administered mice, the mRNA levels of IL-1 β , IL-1 α , IL-2, IL-6, and M-CSF were increased. IL-1 α mRNA was decreased at around a day. In the case of SPG-

Fig. 1. PCR-Assisted Amplification of β -Actin and Cytokine mRNAs from the PEC of OL-2-(Left) and SPG(Right)-Administered ICR Mice
Arrows indicate corresponding PCR product.

Fig. 3. PCR-Assisted Amplification of β -Actin and Cytokine mRNAs from the Liver of OL-2(Left) and SPG(Right)-Administered ICR Mice
Arrows indicate corresponding PCR product.

and AKR mice were done separately, so it is not possible to explain these differences in this study.

DISCUSSION

Hundreds of (1 $\rightarrow$ 3)- β -D-glucans have been screened for their antitumor activity, and two of them, LNT and SPG, have been used clinically.^{1,2)} During the development of these glucans, many studies were carried out and many mechanisms of immunomodulation, especially beneath the cellular level, have been proposed. However, molecular biological characterizations of the activities have not been fully elucidated.

Studies of OL-2, which has two branches in every three main chain units, have helped to clarify the structure-activity relationship dependent on the degree of branching.¹⁷⁾ We have already shown that the pattern of the antitumor action of OL-2 is noteworthy.¹⁰⁾ OL-2 has not shown antitumor activity against the solid form of Sarcoma 180 by i.p. administration, but it has shown such activity against the solid tumor by i.l. administration and against the ascites tumor. Antitumor activity against the ascites tumor has been suggested to be mediated by the activation of phagocytes such as polymorphonuclear leukocyte and macrophage, because OL-2 enhanced the

hematopoietic response by an acute inflammatory response.¹⁰⁾ In contrast, the mechanism of the antitumor activity of glucans against the solid form is mainly by the induction of a tumor specific immune response.¹⁸⁾ The low antitumor activity of OL-2 is thus dependent on the failure of the induction of tumor specific immunity. Findings that OL-2 does not exhibit adjuvant activity to SRBC strongly support this concept.

Analyzing the cytokine gene expression of ICR mice, when comparing OL-2 and SPG administration, a significant difference in the gene expression was observed in some of the cytokines. IL-1ra is a naturally occurring antagonist of IL-1 α and IL-1 β as it binds to type I and type II IL-1 receptors.^{19,20)} OL-2 strongly induced the gene expression of IL-1ra, but SPG did not. The balance between the amount of secretion of IL-1 and IL-1ra may be critical in the immunopharmacological activity of OL-2.

It is well known that glucans activate the haematopoietic system and the activity is pharmacologically important for immune suppressive cancer therapy and microbial infections. In the cytokines modulating the haematopoietic system, OL-2 significantly induced the mRNA of G-CSF and GM-CSF, and SPG significantly induced the mRNA of IL-3. These facts suggested that the mechanism of activation of the hematopoietic system by OL-

Fig. 4. PCR-Assisted Amplification of β -Actin and Cytokine mRNAs from the PEC (Left), Spleen (Center), and Liver (Right) of OL-2-Administered AKR Mice

Arrows indicate corresponding PCR product.

2 and SPG would be different.

Two types of helper T cell clones have been recognized in the mouse on the basis of a differential pattern of cytokine production.²¹⁾ TH1 cells produce IL-2, IFN- γ , and lymphotoxin, and TH2 cells produce IL-4, IL-5, and IL-6. Both of the cells produce IL-3, GM-CSF, and TNF- α . We showed that both OL-2 and SPG enhanced the gene expression of IL-2, but only SPG enhanced that of IL-3. Enhanced IL-2 expression suggested activation of the TH1 cells by both glucans. Guba *et al.* suggested that IL-2 and IL-3 gene expressions required different stimuli.²²⁾ Budd *et al.* suggested that precursor T cells secrete IL-2 but little or no IFN- γ , IL-4, and IL-5, and memory T cells, identified by the Pgp1 cell surface marker, synthesized a larger amount of IFN- γ compared to unprimed T cells.²³⁾ IL-3 was also suggested to be highly synthesized by activated T cells.²¹⁾ These facts suggested that SPG would activate TH1 cells stronger than would OL-2.

IL-3 is a cytokine found in activated helper T cell supernatant that stimulates the growth and differentiation of pluripotent hematopoietic stem cells and leads to the production of all the different blood cell types, including macrophages, granulocytes, megakaryocytes, erythrocytes, eosinophils, and mast cells. Because the cause of the difference of phenotypes of haematopoietic responses between OL-2 and SPG remain unknown, precise

characterization would be of great value.

The pattern of the cytokine gene expression was different in the PEC, spleen, and liver in the cases of both OL-2 and SPG. The kinetics of gene expression were also different in each organ and by each glucan. These characteristic features of the gene expression would relate both to the distribution of glucan²⁴⁾ and to the soluble factors released from the organs by the glucan.

It is generally accepted that protein synthesis, the production of cytokines, and gene expression are differently regulated. For example, TNF α was produced *in vitro* by a glucan treatment, while no TNF was produced *in vivo*, and only the gene expression was observed. It is of interest to clarify the production of cytokines in serum.

The mRNA levels in organs and cells are controlled by rates of both synthesis and degradation. Changes in the cell population in an organ by the accumulation and release of the cells would strongly affect the mRNA level. Thus, a kinetic experiment is important to fully characterize glucan-mediated cytokine gene expression. In this paper we used the RT-PCR method to demonstrate the gene expression of cytokines by i.p. administration of β -glucans. We showed that the mRNA of various cytokines were elevated by glucan administration. It was also found that the kinetics of each cytokine gene expression was dependent on the type of cytokine and the organ. These changes would be related to both the accumulation of

phagocytes, granulocytes, and lymphocytes in these organs and the activation of these cells. It is also important that many kinds of cells, other than the traditional immune cells, could produce cytokines which modulate immune functions. It is known that biological responses modifiers (BRM), including glucan, are multi-cytokine inducers; thus, using the RT-PCR method would be the best way to characterize various cytokine levels in the same sample preparation. It is also important to quantitate the concentration of mRNA levels; however, the RT-PCR method is not sophisticated enough for such quantitation. The addition of an internal standard in the RT-PCR method or monitoring of the concentration of PCR product during the reaction would allow quantitation of the mRNA level. In order to demonstrate glucan-mediated cytokine gene expression more precisely, either of these approaches would be necessary.

We measured the kinetics of cytokine mRNA expression, and thus has to handle many sample preparations at once. We compared the gene expression of cytokines by using the β -actin gene as an external standard. As described in the results, the adjustment of cDNA concentration of each sample was troublesome. It was only possible for each experiment; thus, the result of different experiments could not be directly compared. Some of the RT-PCR products show multiple bands, which would be related to contamination of the undegraded DNA portion, because we did not use DNase to prepare total RNA because of the number of samples. These problems must also be overcome by modifying the RT-PCR method, such as using an internal standard or monitoring the PCR product.

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