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Comparative Study of Immune-Enhancing Activity of Crude and Mannoprotein-Free Yeast-Glucan Preparations

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Abstract β-Glucan, one of the major cell wall components of *Saccharomyces cerevisiae*, is known to enhance the immune function, especially by activating macrophages. Accordingly, in an effort to develop a safe and efficient immune stimulatory agent, we prepared crude β-glucan (glucan-p1) and partially purified β-glucan that was free of mannoproteins (glucan-p2), and evaluated their effect on both the macrophage function and resistance to *E. coli*-induced peritonitis. To investigate the function of the macrophages, phagocytosis, TNF-α secretion, oxygen burst, and the expression of cytokine genes such as IFN-γ and IL-12 were analyzed. Glucan-p2 markedly stimulated the macrophages with all these parameters. Glucan-p1, however, did not stimulate phagocytosis, yet it induced TNF-α secretion, oxygen burst, and the expression of IFN-γ and IL-12, although less efficiently than glucan-p2. Finally, to test the *in vivo* protective effect of β-glucan against infection, the survival of mice from *E. coli*-induced peritonitis was investigated. After 24 h of the peritoneal challenge of *E. coli*, all of the mice treated with glucan-p2 survived whereas none survived in the control group. Glucan-p1 showed only a marginal effect in protecting the mice. These results suggest that mannoprotein-free glucan-p2, but not glucan-p1, can serve as an effective immune-stimulating agent.

Key words: β-Glucan, macrophage, immune-stimulatory agent, TNF-α, oxygen burst, phagocytosis, IL-12, IFN-γ, *E. coli*-induced peritonitis

β-Glucan is a polymer of β-D-glucose connected through the β-(1→3) main chain and β-(1→6) side chain glycosidic linkages. It represents a major structural component of most fungal cell walls including *S. cerevisiae* [10, 16]. A fungal cell wall consists of polysaccharides and glycoproteins. Polysaccharides appear to have a structural function, whereas the mannoproteins seem to act as a filler. The polysaccharides of the yeast cell wall are composed of β-glucan (60%) and β-mannan (20%).

β-Glucan is known to possess antitumor and antimicrobial activities by enhancing the host immune function [6, 7]. β-Glucan activates macrophages, neutrophils, and NK cells by binding to a β-glucan receptor on these cells. Among these, macrophages are the best characterized target of β-glucan [2, 15]. The receptor of β-glucan was recently identified as CR3 (C-receptor type 3)(Mac-1, CD11b/CD18), a known receptor of iC3b, ICAM-1, and fibrinogen. The β-Glucan binding site (lectin site) of CR3 has been mapped to the I-domain of CD11b [5, 20]. However, it is not clear whether CR3 is the only receptor of β-glucan. Binding β-glucan to its receptor triggers phagocytosis, respiratory bursts, and the secretion of cytokines such as TNF-α [4, 21]. Thus, β-glucan is a promising candidate for an immune-stimulatory agent for immune-compromised patients or for infections by multidrug-resistant bacteria. There is also evidence that β-glucan may be applicable to tumor immunotherapy. The side effects of β-glucan such as hepatosplenomegaly, microembolization, and increased endotoxin sensitivity are likely due to low solubility and contaminating residual proteins [1]. Therefore, an optimal procedure for yeast β-glucan preparation needs to be developed in addition to further understanding of the immune-enhancing mechanism of β-glucan on the cellular and molecular levels. Accordingly, an alkali-soluble β-glucan (glucan-p1) was prepared from the cell wall of *S. cerevisiae* and purified further by removing residual proteins in the form of mannoprotein complexes (glucan-p2). Thereafter, these two preparations of β-glucan were compared for their effects on macrophages *in vitro*

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and protection of mice against *E. coli*-induced peritonitis. The results showed that mannoprotein-free β-glucan enhanced the function of macrophages and protected mice from lethal peritonitis induced experimentally by *E. coli*.

MATERIALS AND METHODS

Cell Culture

A murine macrophage cell line, Raw 264.7, and a human fibroblast cell line, L929, were purchased from ATCC (Rockville, U.S.A.) and Korea Cell Line Bank (KCLB) (Seoul, Korea), respectively. The cells were grown in an RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, endotoxin tested, Gibco BRL, Grand Island, U.S.A.), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES (N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid).

Preparation of β-Glucan Samples

The cell wall of *S. cerevisiae* was obtained from the Choheung Chemical Company (Ansan, Korea). β-Glucan was extracted from the yeast cell wall in a 2% (w/v) NaOH solution at 90°C for 5 h. After cooling, the suspension was centrifuged at 3,000 ×g for 10 min. The supernatant was neutralized with 2 M acetic acid and β-glucan was precipitated by adding 3 volumes of ethanol. The precipitate was washed with ethanol and freeze-dried (glucan-p1). The glucan-p1 was dissolved in 3% (v/v) acetic acid and centrifuged to remove any insoluble β-glucan. The recovered supernatant was then neutralized with 2 M NaOH. The recovered β-glucan still contained a significant amount of proteins, usually in mannoprotein complexes. To remove these residual proteins, the β-glucan sample was loaded onto DEAE sephacel (Pharmacia Biotech, Uppsala, Sweden) anion-exchange column. The column was washed with 10 mM sodium phosphate buffer (pH 8.0) and 3 bed-volumes of the unbound fraction were collected. The β-Glucan in the unbound faction was precipitated with 90% ethanol, dialyzed against double distilled water, and freeze-dried. The resulting β-Glucan was solubilized in 5% glucose solution (glucan-p2). The concentration of the residual protein in each step was measured by the Bradford method [25].

Peritoneal Macrophages

ICR mice were sacrificed by cervical dislocation and the peritoneal macrophages were obtained by washing out the peritoneal cavity with 1×PBS (phosphate-buffer saline). The cells were resuspended in a serum-free RPMI 1640 medium and plated on a 96-well culture plate at $3-5\times10^5$ cells/well. After a 2 h incubation at 37°C, the nonadherent cells were removed and used within 24 h.

TNF-α Bioassay

The TNF-α secretion was determined using a modified L929 cytotoxicity assay [13]. The L929 cells in RPMI-1640 with 10% FBS (4×10^4/well) were plated on a 96-well culture plate and incubated overnight. On the next day, the medium was replaced with RPMI-1640 containing actinomycin D (1 μg/ml). The samples were added to the L929 cells in triplicate and the plates were further incubated for 18 h at 37°C. After incubation, the cells were stained with 0.5% crystal violet and the absorbance at 490 nm was determined (MRX Microplate Reader, Dynatech, Chantilly, U.S.A.). Different concentrations of recombinant murine TNF-α (PharMingen, San Diego, U.S.A.) were used as standards.

Phagocytosis Assay

Mouse peritoneal macrophages (3×10^5/well) in a 96-well plate were treated with 1 mg/ml of β-glucan for 24 h. FITC-labeled *E. coli* or *S. aureus* (6×10^6/well) (Molecular Probes, Eugene, U.S.A.) was added to the macrophages. After various periods of incubation, the supernatant was removed by aspiration. Tryphan blue (100 μl, 250 μg/ml, pH 4.4) was added to each well and incubated for 1 min to quench any extracellular fluorescence. The macrophages were then lyzed by adding 100 μl of 0.2% SDS in a CBS buffer (70 mM $NaCO_3$, 930 mM $NaHCO_3$, and 154 mM NaCl, pH 9.0). The phagocytosis of the bacteria was analzyed by reading the fluorescence intensity in the cell lysates at 480±10 nm excitation and 530±12.5 nm emission wavelengths using an automated fluorescence microplate reader (HTS 7000 Bioassay reader, Perkin Elmer, Norwalky, U.S.A.). The fluorescence intensity was expressed as a relative fluorescence unit (RFU).

Chemiluminescence (CL) Assay

The oxidative metabolic activity of the macrophages was determined by a luminol-dependent CL assay using a Biolumat LB 9505 (Berthold, Bad Wildbad, Germany). To 1×10^5 cells of Raw 264.7 in 100 μl of PBS, 5 μl of 10 mg/ml luminol (5-amino-2,3-dihydro-1,4 phthalazinedione) and 890 μl of a KRP buffer (0.1 M $NaHPO_4$, 0.9% NaCl, 0.154 M KCl, and 0.154 M $MgCl_2$) were added. The samples were incubated for 5 min in a 37°C waterbath and then 5 μl of phorbol myristate acetate (PMA, 0.1 mg/ml) was added. CL was measured at 40-60 second intervals. The CL was measured in the relative light unit (RLU) obtained using the rate mode. All experiments were performed in triplicate.

RNA Extraction and RT-PCR

Total cellular RNA was extracted using the acid guanidium isothiocyanate method [3]. cDNA was synthesized at 37°C for 60 min in a mixture containing total RNA (5 μg), 1 mM of all 4 dNTPs, 10 μg/ml oligo (dT) primer (Promega, Madison, U.S.A.), 0.6 U/μl RNasin (Promega, Madison, U.S.A.), 2 mM $MgCl_2$, and 6 U/μl MMLV-RT

Table 1. Protein contents after each purification step.

Purification Step	Residual Protein Contents (%, w/w)	Recovery Yield (%, w/w)
NaOH Extraction (glucan-p1)	2.8	31
Acetic Acid Dissolution	0.8	-
DEAE Ion-Exchange Chromatography (glucan-p2)	0.3	13

(Gibco BRL, Grand Island, U.S.A.). The PCR mixture (50 μl) consisted of cDNA (one tenth of the above RT reaction corresponding to 0.5 μg of the total RNA), 0.5 μM of each primers, 0.2 mM of all 4 dNTPs, 100 mM Tris-HCl, 500 mM KCl, 0.8% Nonidet P40, and 0.05 unit/μl Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania). Thirty three or thirty eight PCR cycles of 1 min denaturation at 95°C, 1 min annealing at 58°C, and 1 min extension at 72°C were carried out. The PCR products were analyzed on a 1.5% agarose gel. The sequences of the primers specific for the mouse IL-12 p40, mouse IFN-γ, and human β-actin genes were as follows: 5'-CCACTC-ACATCTGCTGCTCCACAAG-3' (IL-12 forward), 5'-ACTTCTCATAGTCCTTTGGTCCAG-3' (IL-12 reverse), 5'-TCTGAGACAATGAACGCTAC-3' (IFN-γ forward), 5'-AATACTTGTTTGGTTGATGC-3' (IFN-γ reverse), 5'-CAGAGCAAGAGAGGCATC-3' (β-actin forward), and 5'-CGTAGATGGGCACAGTGT-3' (β-actin reverse).

E. coli-Induced Peritonitis

ICR mice weighing approximately 25 g were challenged with clinically-isolated *E. coli* (1.0×10^8). The *E. coli* challenge was made by injecting 0.1 ml of the bacterial suspension in PBS into the peritoneal cavity. Each experimental group consisted of 5 male mice. To determine the protective effect of β-glucan against *E. coli*-induced peritonitis, the mice were injected intraperitoneally with β-glucan (150 or 500 mg/kg), 5 and 3 days prior to the *E. coli* challenge. Survival was monitored hourly during the first 24 h.

Results and Discussion

Preparation of Yeast β-Glucan

The cell wall of *S. cerevisiae* consists of three layers which are probably merged together in part: an inner layer of alkali-insoluble β-glucan, a middle layer of alkali-soluble β-glucan, and an outer layer of glycoprotein of which the carbohydrate is composed of phosphorylated mannan. To obtain the alkali-soluble β-glucan, the yeast cell wall was extracted with 2% NaOH (glucan-p1). The resulting glucan-p1 contained 2.8% of the protein (Table 1). Dissolution with 3% acetic acid reduced the residual protein content to 0.8%. The β-glucan was further purified through DEAE ion exchange column chromatography to remove any residual proteins (glucan-p2). The protein content of the resulting glucan-p2 was 0.3% (w/w). The final recovery of glucan-p2 was 13% (w/w) (Table 1).

Effect on TNF-α Release from Murine Macrophage Cell Line, Raw 264.7

TNF-α plays a crucial role in host defense by stimulating secretion of other inflammatory cytokines, enhancing vascular endothelial cell permeability and promoting recruitment of additional immune effector cells [24]. In macrophages and neutrophils, TNF-α stimulates oxygen bursts and phagocytic activity which leads to the efficient killing of microbes. Furthermore, TNF-α may also directly affect the viability of certain microorganisms, such as *Pneumocystitis carini* and *trypanosomes* [14].

To test the ability of the two β-glucan preparations in stimulating a TNF-α release, Raw 264.7 cells were treated

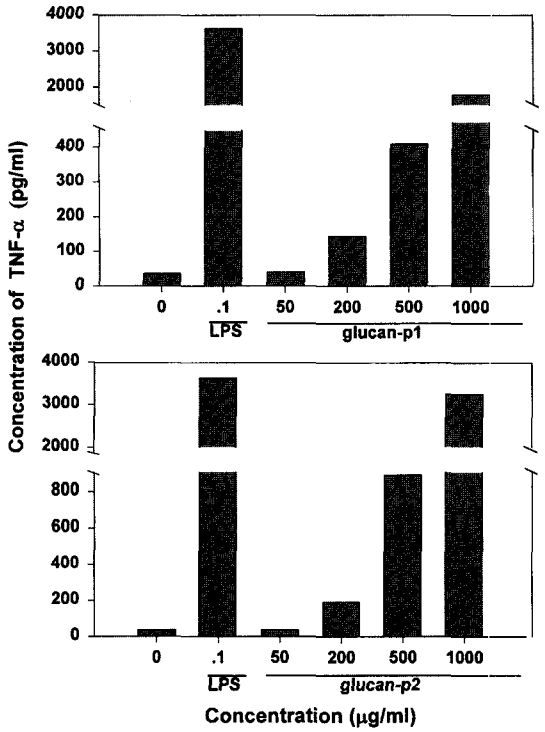

Fig. 1. Effect of β-glucan on TNF-α secretion by Raw 264.7 cells.

β-Glucan was added to Raw 264.7 cells in triplicate at the indicated concentrations and incubated for 24 h. The supernatants were harvested and the resulting concentrations of TNF-α in the supernatants were measured by an L929 cytotoxicity assay. The TNF-α secretions by glucan-p1 and glucan-p2 stimulation are shown in the upper and lower panels, respectively. LPS was used as a positive control.

with β-glucan for 24 h and the culture supernatant was analyzed for TNF-α bioactivity using an L929 cytotoxicity assay. Figure 1 shows that both glucan-p1 and glucan-p2 (50-1,000 μg/ml) induced TNF-α secretion from Raw 264.7 cells in a dose-dependent manner. However, glucan-p2 was approximately 2 times more effective in inducing TNF-α than glucan-p1 at concentrations 500 and 1,000 μg/ml. For example, when cells were stimulated with 1,000 μg/ml of β-glucan, the TNF-α concentrations in the culture supernatants were 1,780 pg/ml for glucan-p1 and 3,240 pg/ml for glucan-p2.

Effect on Phagocytosis of Macrophages

Phagocytosis is one of the most important host defence mechanisms against invading microbes, therefore, phagocytic activity is a critical measurement of the macrophage function [22]. The recognition of microbes can be mediated by receptors for common microbial constituents [8]. The β-glucan receptor, CR3, is one of the receptors used for recognizing microbial pathogens and is responsible for phagocytosis and subsequent degranulation and respiratory bursts.

To investigate the effect of β-glucan on phagocytic activity, the phagocytosis of FITC-labeled *E. coli* or *S. aureus* by mouse peritoneal macrophages was measured.

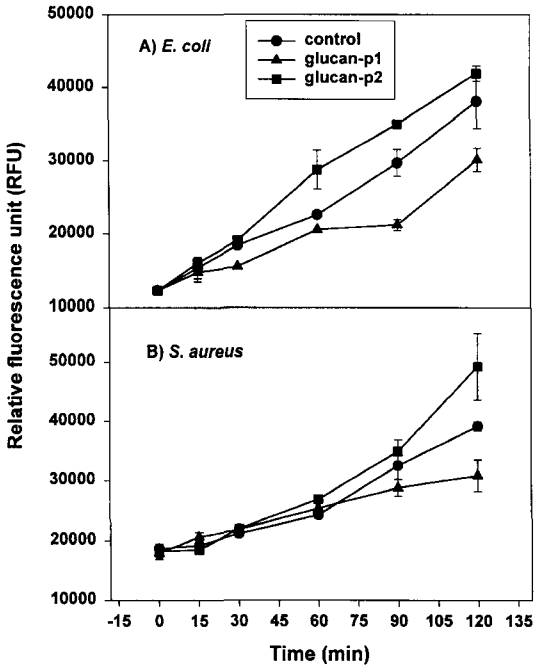

Fig. 2. Effect of β-glucan on phagocytosis by mouse peritoneal macrophages.

Mouse peritoneal macrophages (3×10^5/well) were stimulated with 1 mg/ml of glucan-p1 or glucan-p2 for 24 h in a 96-well plate. After FITC-labeled *E. coli* (A) or *S. aureus* (B) was added to the macrophages at a ratio of 1:20 (macrophage:bacteria), the plate was incubated for up to 120 min. After removing the supernatant, the cells were lyzed and the fluorescence was measured with an automated fluorescence microplate reader.

The effect of β-glucan on phagocytosis showed a similar pattern for both the gram-positive *S. aureus* and gram-negative *E. coli* (Figs. 2A and 2B). The glucan-p2 increased the phagocytic activity of the macrophages slightly yet consistently, whereas glucan-p1 tended to reduce the phagocytosis. These results were reproducable in several independent experiments. However, when the mice were injected with glucan-p1 intraperitoneally, the phagocytic activity of the macrophages from the glucan-stimulated mice substantially increased (data not shown), indicating that glucan-p1 can stimulate the phagocytic activity of macrophages *in vivo* but not *in vitro*.

Effect on Macrophage Oxygen Burst

Upon contact with bacteria or other foreign materials, phagocytic cells generate reactive oxygen intermediates (ROI), such as superoxide anions, hydrogen peroxide, singlet oxygen, and hydroxyl radicals which are generally referred to as oxygen or respiratory bursts [14]. ROI are potent microbicidal agents and important in killing phagocytosed microorganisms. ROI are generated by the NADPH oxidase system which is activated by the perturbation of the plasma membrane during phagocytosis [19]. However, phagocytosis and oxygen bursts appear to be relatively independent processes, because the induction of these two responses are dissociated in some situations [23].

To study the effect of β-glucan on oxygen bursts in macrophages, Raw 264.7 cells were treated with β-glucan for 24 h and stimulated with PMA to induce an oxygen burst. The oxygen burst of the Raw 264.7 cells reached a peak between 5 to 30 min after PMA stimulation and decreased slowly thereafter. The oxygen burst induced by PMA was markedly increased by the stimulation of glucan-p2 (Fig. 3). Glucan-p1 showed only a slight increase

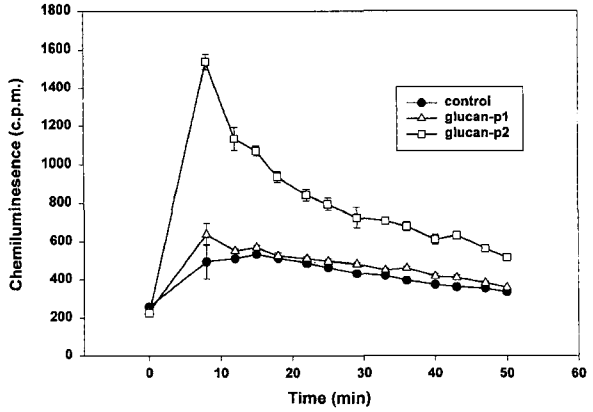

Fig. 3. Effect of *in vitro* β-glucan stimulation on oxygen burst of Raw 264.7.

Cells were incubated with 1 mg/ml β-glucan for 24 h. An oxygen burst was induced by PMA and the resulting luminol-dependent chemiluminescence was measured as described in Materials and Methods. All experiments were performed in triplicate.

over the control. Thus, the *in vitro* treatment of glucan-p2 significantly augmented the generation of oxygen metabolites by macrophages. Similar to phagocytosis, the *in vivo* treatment of glucan-p1 enhanced the oxygen burst from the peritoneal macrophages (data not shown).

Effect on IL-12 and IFN-γ Expression in Macrophages

IL-12 is produced by activated macrophages and dendritic cells, and is a potent inducer of IFN-γ by T cells and NK cells [9, 17, 18]. IL-12 and IFN-γ are the principal mechanisms linking innate and adaptive immunity to a variety of intracellular infections. During the initial phase of infection, IL-12 activates NK cells and promotes the development of specific immunity by enhancing differentiation of IFN-γ producing T_H1 cells. IFN-γ is a potent activator of macrophages and enhances IL-12, and promotes the maintenance of a T_H1-type response [12]. Therefore, IL-12 and IFN-γ can have a profound effect on a host resistance to intracellular infection and malignant tumors.

The induction of T_H1-related cytokines, IL-12, and IFN-γ, in mouse peritoneal macrophages by β-glucan was analyzed at the RNA level using RT-PCR. Mouse peritoneal macrophages were treated with β-glucan for 2 or 16 h. As shown in Fig. 4, IL-12 and IFN-γ mRNA were induced by both glucan-p1 and glucan-p2. The expression of IL-12 was substantially increased at 2 h when treated with glucan-p2. Glucan-p1 also induced IL-12 expression, yet, when compared to glucan-p2, the induction was weaker and slower. IFN-γ expression was increased by glucan-p2 treatment at 2 h and further increased at 16 h. The increase of IFN-γ production by glucan-p1 was smaller than that by glucan-p2. Overall, glucan-p2 was more effective than glucan-p1 in inducing both IL-12 and IFN-γ mRNA expression.

Intracellular signaling pathways by which β-glucan triggers in macrophages is largely unknown. Since IFN-γ is a potent stimulator of macrophages, it is possible that the enhancing effect of β-glucan on the macrophage function is, at least in part, mediated by IFN-γ in an autocrine fashion. However, the extent by which the macrophage-stimulating effect of β-glucan is mediated by IFN-γ is not presently known.

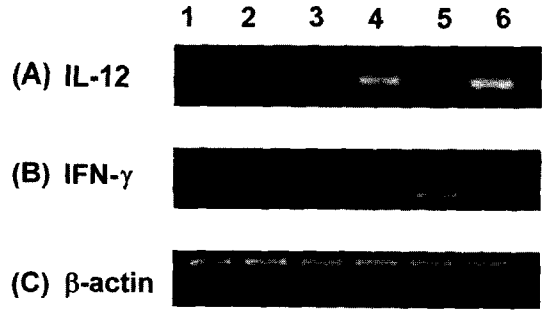

Fig. 4. Effect of β-glucan on IL-12 and IFN-γ expression of mouse peritoneal macrophages.

Mouse peritoneal macrophages were stimulated with β-glucan (1 mg/ml) for 2 or 16 h. The preparation of total cellular RNA and RT-PCR was performed as described in Materials and Methods. Lane 1, control; lane 2, glucan-p1 treatment for 2 h; lane 3, glucan-p1 treatment for 16 h; lane 4, glucan-p2 treatment for 2 h; lane 5, glucan-p2 treatment for 16 h; lane 6, LPS treatment for 2 h.

Effect of β-Glucan on Survival of Mice with *E. coli*-Induced Peritonitis.

The *in vivo* immune-stimulatory effect of β-glucan was tested by measuring protection of mice from *E. coli*-induced peritonitis. Mice were injected with β-glucan 3 times, 1, 3, and 5 days prior to the *E. coli* challenge (1.0×10^8). The kinetics of death were recorded during the first 24 h after the *E. coli* challenge. Twenty % of the mice in the group pretreated with glucan-p1 (500 mg/kg) survived, whereas the group pretreated with glucan-p2 (75 mg/kg or 150 mg/kg) showed 100% survival rates. All of the mice in the control group, which were treated with 5% glucose, died within 24 h (Fig. 5). These results clearly show that glucan-p2 can protect mice from *E. coli*-induced peritonitis. It appears that the *in vitro* macrophage activation by β-glucan correlated with the *in vivo* protection from *E. coli*-induced peritonitis, although β-glucan activated neutrophil and NK cells in addition to macrophages, and might also enhance specific immunity.

In summary, glucan-p2 possess greater immune enhancing effects than glucan-p1 *in vitro* and *in vivo*. Two mechanisms may explain why glucan-p2 shows higher immune stimulatory activity compared to glucan-p1. First, glucan-p2 has a higher glucan content than glucan-p1, since glucan-p1 contains proteins which are removed during DEAE Sephacel column chromatography from glucan-p2. Second, more

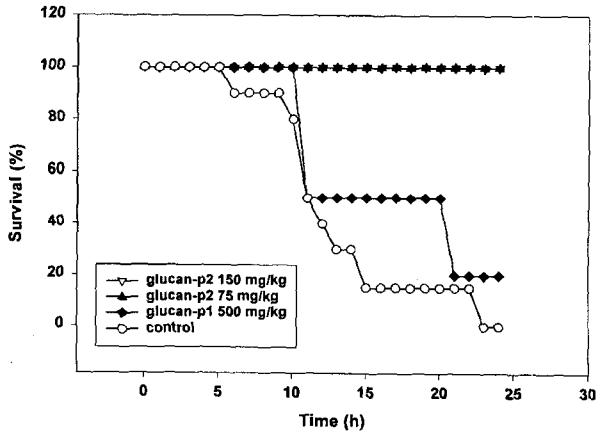

Fig. 5. Protective effect of β-glucan on *E. coli*-induced peritonitis.

Glucan-p1 (500 mg/kg) or glucan-p2 (75 mg/kg or 150 mg/kg) was administered intraperitoneally 3 times, 5, 3, and 1 days prior to the *E. coli* (1×10^8/mouse) challenge. As a control, 5% glucose was administered instead of β-glucan. The mice were monitored for 24 h and the kinetics of death were recorded.

importantly, the contaminating proteins in glucan-p1 may exert inhibitory effects on the immune function. For example, glucan-2 increases proliferation of mouse spleen cells *in vitro*, whereas glucan-p1 does not (data not shown). This may not simply be due to the lower β-glucan content in glucan-p1, because proliferation of spleen cells is somewhat increased at lower concentrations of glucan-p1.

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