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Immunopharmacological Activity of the Purified Insoluble Glucan, Zymocel, in Mice

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Abstract

Although it has been established that soluble glucan in fungi is important to host defence against infection, the importance of insoluble glucans is not clear. We have examined the in-vivo immunopharmacological activity of the insoluble glucan, zymocel.

Administration of zymocel increased peritoneal exudate cell number and spleen weight, and enhanced: phagocytic activity, hydrogen peroxide production, and nitric oxide production of peritoneal exudate cells; the extravascular release of Evans blue (which might reflect vascular permeability); lipopolysaccharide-triggered synthesis of tumour necrosis factor (TNF); and recovery of white blood cell number in cyclophosphamide-induced leukopenia. Zymocel also showed anti-tumour activity against sarcoma 180 in mice and also enhanced TNF synthesis and hydrogen peroxide production by macrophage-like cell line in-vitro, i.e. resulted in direct macrophage activation.

These results show that zymocel shows varied immunopharmacological activity; it is suggested that the administration of insoluble glucan induces the inflammatory response, the subsequent activation of the immune systems via the cytokine network, and direct macrophage activation.

Infectious fungi contain polysaccharides such as glucans and mannans and that some of these interact with complement components resulting in activation of the complement system both by classical and alternative pathways (Suzuki et al 1992). It is thought that one of the initial steps of the recognition of fungi could be binding with mannan binding protein, mammalian lectin, in serum. The complex formed activates the classical complement pathway (Lu et al 1990), and the corresponding immune system, e.g. phagocytosis by macrophages. The presence of a mannose receptor specific for mannan has been reported (Warr 1980), and it is suggested that this receptor is important in the exclusion of fungi from the body (Schlesinger 1993). Although the glucan entity is also thought to be important in inducing inflammatory response, a glucan-specific receptor has not yet been clearly demonstrated. The glucan portion could be important in the pathophysiology of the fungi, both in acute and chronic stages. The role of these polysaccharides in the infection of fungi and their pharmacological activities are, however, not yet clearly defined.

Soluble glucans, such as lentinan and schizophyllan (SPG), show a variety of biological activity, e.g. antitumour activity, macrophage activation, complement activation, etc. (Taguchi et al 1988; Fujimoto et al 1988). It has been established that soluble glucan is important to the mechanism of robust defence by the host. Although a mixture of soluble and insoluble glucans exist in fungi, differences between the pathophysiological and pharmacological activity of these forms are not clear.

Zymosan, a glucan-rich particulate material derived from yeast, has been used for in-vitro and in-vivo studies (Abel 1992; Rao et al 1994) and highly purified particulate glucan, zymocel, prepared from a yeast, has recently become commercially available. It is of interest to compare the immunopharmacological activity of zymocel with that of the soluble glucans. This paper describes an examination of the immunopharmacological activity of zymocel and discusses the spectrum of activity with soluble glucans.

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Materials and Methods

Materials

Zymosan A (zymosan), phorbol myristate acetate, scopoletin, horseradish peroxidase and zymocel were purchased from Sigma (St Louis, MO, USA). Cyclophosphamide was from Wako Pure Chemical Industries Ltd, (Osaka, Japan). RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan), supplemented with penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}) and 5 mM HEPES (Sigma), was used for cell culture. SSG, a β-glucan obtained from a fungus, *Sclerotinia sclerotiorum* was prepared in our laboratory.

Animals

Specific-pathogen-free male ICR mice were purchased from Japan SLC (Shizuoka, Japan) and used at 5–10 weeks of age. Mice were housed under specific-pathogen-free conditions.

Measurement of the number of peritoneal exudate cells

Cells were collected from the peritoneal cavities of mice by washing the cavities twice with fresh Hank's balanced salt solution (5 mL) containing heparin (5 units mL^{-1}). The cells were washed twice and resuspended in RPMI 1640 medium. The total number of cells was counted with a haemocytometer.

Measurement of vascular permeability

Stimulant (1 mg/mouse), such as zymocel, was administered to mice by intraperitoneal injection. Soon after injection an

aqueous solution of Evans blue (Sigma E-2129; 10 mg mL^{-1} in saline; 0·2 mL) was injected intravenously and 30 min later the mice were killed and the peritoneal exudate fluids were collected by lavage with phosphate-buffered saline (4 mL). After centrifugation to remove any debris the concentration of Evans blue in the peritoneal cavity was measured at 630 nm.

Isolation of peritoneal macrophages

Peritoneal macrophages were isolated from the peritoneal cavity by lavage with 2 washes of 5–6 mL Hanks balanced salt solution containing 1% heat-inactivated foetal calf serum, 5 mM HEPES, 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. The cells were cultured in 96-well flat-bottomed plates at 2×10^5 cells per well in 100 mL culture medium. To ensure adherence cells were cultured for at least 2 h before washing and washed twice with fresh medium (50 μL/well).

Preparation of macrophages

The mouse macrophage-like cell line, RAW264.7 (Riken Cell Bank, Tsukuba, Japan) was cultured to confluence in RPMI1640-10% foetal calf serum. RAW264.7 cells were suspended at a cell density of $2{\cdot}5 \times 10^6$ cells mL^{-1} in RPMI1640-10% foetal calf serum. RAW264·7 cells were stimulated with or without GRN in a humidified 5% CO_2 incubator. After incubation, culture supernatant was collected by centrifugation at 300 *g* for 5 min. Macrophages adhering to the culture plate were lysed in distilled water by repeated freezing and thawing (three times) then addition of a twofold concentration of RPMI1640 and filtration through a syringe filter unit (0·2 μm, Corning).

Measurement of H_2O_2 production by peritoneal macrophages

Production of H_2O_2 was assayed continuously at 37°C by micro-assay using the scopoletin technique. Briefly, the cultures were washed (3×50 μL) with Krebs-Ringer phosphate buffer supplemented with 5·5 mM glucose (KRPG). The assay mixture consisting of 30 mM scopoletin, 1 mM NaN_3, 1 purpurogallin unit (mL horseradish peroxidase)$^{-1}$ and 100 ng mL^{-1} phorbol myristate acetate in KRPG was replaced in each well. Immediately after the addition of the assay mixture the plate was placed in a filter fluorimeter (microplate reader MTP-32; Corona Electric, Tokyo, Japan) and the fluorescence was recorded with an excitation wavelength of 365 nm and an emission wavelength of 450 nm. After incubation for 120 min at 37°C, the fluorescence in each well was again recorded using the microplate reader. The amount of H_2O_2 produced by macrophages was calculated from the fluorescence reduction using H_2O_2 solution as standard.

Phagocytosis assay

Peritoneal macrophages prepared on the slide glass were washed three times with fresh medium. Zymosan (5×10^5 particles per well) was added to peritoneal macrophages, and incubated at 37°C for a further 30 min. The cells were washed three times with fresh medium, fixed with methanol, and stained with Diff-Quik stain. The number of phagocytic cells ingesting zymosan out of a total of at least 200 cells were determined by direct visual enumeration by means of a light microscope (400 ×).

Measurement of nitric oxide production by peritoneal exudate cells

Nitric oxide, quantified by the accumulation of nitrite in the culture medium, was measured spectrophotometrically, using the Griess reaction with sodium nitrite as the standard. Briefly, culture supernatant (48-h culture; 100 μL) was mixed with sulphanilamide (1%; 100 μL) and *N*-1-naphthylethylenediamine dihydrochloride (0·15%) in 2.5% H_3PO_4. After 10 min at room temperature the optical density (OD) was measured at 550 nm.

Preparation of mouse serum

The mouse blood was obtained by cardiopuncture 1 h after lipopolysaccharide injection. The blood was left to clot at room temperature for 1 h and at 4°C for 30 min. The blood was then centrifuged at 15 000 rev min^{-1} for 5 min and the supernatant was used as a mouse serum sample for assay of tumour necrosis factor α (TNF-α).

TNF-α assay

Serum TNF-α level was detected by an indirect enzyme-linked immunosorbent assay (ELISA). Anti-mouse TNF-α mAb (Pharmingen, San Diego, CA, USA; 5 μg mL^{-1}) was bound to the surface of 96-well, flat bottomed plates (Sumitomo Bakelite, Tokyo, Japan) by incubating at 4°C overnight in bicarbonate buffer (0·1 M; pH 9·6). The plate was washed with phosphate-buffered saline containing 0.05% tween 20 (PBST) and blocked with 0.5% bovine serum albumin in PBST at 37°C for 40 min. The plate was washed with PBST and incubated with rMu TNF-α (R & D Systems, Minneapolis, MN, USA) or the samples at 37°C for 40 min. The plate was washed and then treated with a 1 : 2000 dilution of anti-TNF-α rabbit polyclonal antibody (Genzyme, Boston, MA, USA). After washing and blocking the plate was treated with a 1:10 000 dilution of peroxidase-labelled anti-rabbit immumoglobulin G (IgG; Genzyme, Boston, MA, USA). After final washing, peroxidase-conjugated antibody was detected by the addition of a (TMB) 3,3′,5,5′-tetramethylbenzidine substrate system (KPL, Gaitherburg, MD, USA.). Colour development was stopped with 1 N phosphoric acid and the absorbance at 450 nm was measured with the MTP32 microplate reader.

Evaluation of antitumour activity

Antitumour activity was evaluated against the solid form of sarcoma 180 tumour cells. Tumour cells (5×10^6) were inoculated subcutaneously into the right groin of ICR mice. Zymocel was administered intraperitoneally or intravenously to ICR mice 7 days after tumour inoculation. The antitumour activity was assessed by percent inhibition, which was calculated as: [1-(average tumour weight of the treated group/ average tumour weight of the control group)] × 100 (%).

White blood cell number

Blood (5 μL) collected from the tail vein was mixed with Turk's solution and counted by use of a haemocytometer.

Results

Peritoneal exudate cell number and spleen weight of zymocel-administered mice

The peritoneal exudate cell number and spleen weight of zymocel-treated mice were examined on day 5. Administration of zymocel increased peritoneal exudate cell number in a dose-depended manner (Table 1). Administration of zymocel (250 μg) significantly enhanced spleen weight ($P < 0.05$).

Effects of zymocel on vascular permeability

Because acute inflammation is often accompanied by increased vascular permeability, vascular permeability in mice treated with insoluble glucan was assessed by measuring the extravascular release of Evans blue. As shown in Table 2, zymocel and zymosan significantly released Evans blue into the peritoneal cavity within 30 min ($P < 0.001$). This strongly suggested the induction of an acute phase response.

Effect of zymocel administration on synthesis of H_2O_2 by peritoneal macrophages

Phorbol myristate acetate-triggered H_2O_2 production by zymocel- or zymosan-elicited peritoneal macrophages was examined on days 1, 3 and 7. The macrophages collected on days 1 and 3 significantly elevated phorbol myristate acetate-triggered H_2O_2 synthesis. For zymosan, on the other hand, maximum H_2O_2 production was observed on day 1, and the activity decreased thereafter (Table 3).

Effect of zymocel administration on phagocytosis of zymosan particle by peritoneal macrophages

In addition to H_2O_2 production, phagocytic activity of peritoneal macrophages was examined after administration of zymocel or zymosan. Phagocytic activity of zymocel- and zymosan-elicited macrophages was significantly increased in comparison with that of the saline-treated group (Table 3).

Effect of zymocel administration on nitric oxide synthesis of peritoneal exudate cells

Nitric oxide, an important mediator produced by macrophages, directly kills tumour cells and denatures cells and microorganisms. Nitric oxide could be a key molecule in biological-response-modifier-mediated activity. We have previously reported that a soluble glucan, schizophyllan, enhanced nitric oxide synthesis by peritoneal exudate cells. Table 4 shows that nitric oxide synthesis of peritoneal exudate cells collected from mice treated with zymocel was enhanced in a dose-dependent manner.

Priming effect of zymocel on in-vivo lipopolysaccharide-triggered TNF synthesis

Intravenous or intraperitoneal injection of zymocel had a dose-dependent priming effect on lipopolysaccharide-triggered TNF-α synthesis. The activity of zymocel was, however, less than that of SSG, a soluble β-glucan (Table 5).

Table 1. Peritoneal exudate cell number and spleen weight of mice treated with zymocel.

	Peritoneal exudate cell number ($\times 10^6$/mouse)	Spleen weight (g)
Control	2·45 ± 0·77	0·165 ± 0·018
Zymocel 50 μg	3·18 ± 1·18	0·167 ± 0·028
Zymocel 250 μg	7·67 ± 0·60*	0·267 ± 0·058†

Zymocel was administered to mice intraperitoneally; peritoneal exudate cell number and spleen weight were measured on day 5. *$P < 0.01$, †$P < 0.05$.

Table 2. Extravascular release of Evans blue after the administration of zymocel.

	Optical Density at 630 nm
Saline	0·12 ± 0·01
Zymosan	0·53 ± 0·12*
Zymocel	0·49 ± 0·19*

The stimulant and Evans blue solution were administered to mice intraperitoneally and the concentration of Evans blue in the peritoneal cavity was measured 30 min later. Results are means ± s.d. *$P < 0.001$.

Table 3. Effect of particulate glucans on phorbol myristate acetate-induced H_2O_2 production and phagocytic activity of zymosan by murine peritoneal macrophages.

		Cell number ($\times 10^6$ mL^{-1})	Phorbol myristate acetate-induced H_2O_2 production (μM)	Phagocytosis (%)
Day 1	Zymosan	11·6 ± 1·1	13·21 ± 0·45	65·41 ± 3·41*
	Zymocel	12·6 ± 3·5	5·63 ± 0·76	74·29 ± 0·84†
	Saline	2·2 ± 0·8	0·58 ± 0·26	53·32 ± 0·64
Day 3	Zymosan	8·5 ± 2·0	2·98 ± 0·90	56·45 ± 2·50*
	Zymocel	11·4 ± 1·4	6·33 ± 0·33	58·75 ± 0·23
	Saline	2·2 ± 1·4	1·06 ± 0·56	37·08 ± 5·08
Day 7	Zymosan	2·8 ± 0·0	2·96 ± 0·34	58·36 ± 2·57‡
	Zymocel	8·4 ± 0·0	N.D.	57·74 ± 4·23*
	Saline	3·6 ± 0·0	0·34 ± 0·00	46·05 ± 1·59

Particulate glucan (250 μg/mouse, i.p.) was administered to mice and macrophages were collected on days 1, 3 and 7. Adherent macrophages were stimulated with phorbol myristate acetate (100 ng mL^{-1}) for 120 min. Released H_2O_2 was estimated from the reduction of fluorescence intensity of scopoletin. For phagocytosis assay, macrophages were incubated with zymosan (200 μg mL^{-1}) for 30 min. Phagocytosis shows the percentage of 200 cells that injested at least one zymosan particle. *$P < 0.05$, †$P < 0.005$, ‡$P < 0.01$.

Table 4. Induction of NO synthesis by peritoneal exudate cells after administration of zymocel.

	Concentration of nitric oxide (μM)
Control	11 ± 9
Zymocel 50 μg/mouse	26 ± 13*
Zymocel 250 μg/mouse	40 ± 7*

Peritoneal exudate cells were collected from the peritoneal cavity after administration of Zymocel. NO was measured spectrophotometrically using the Griess reaction with sodium nitrite as standard. Results are shown as the mean ± s.d. *$P < 0.001$.

Table 5. Priming effect of zymocel on lipopolysaccharide-triggered TNF-α synthesis in-vivo.

	Route	TFN-α titre (ng mL^{-1})
Control	—	8 ± 5
Zymocel 50 μg/mouse	Intraperitoneal	18 ± 14
Zymocel 250 μg/mouse	Intraperitoneal	28 ± 13†
Zymocel 50 μg/mouse	Intravenous	16 ± 13
Zymocel 250 μg/mouse	Intravenous	27 ± 22*
SSG	—	47 ± 26†

Lipopolysaccharide (10 μg) was administered to mice 10 days after administration of zymocel or SSG. TNF-α concentration in serum was measured by ELISA using anti-TNF-α antibodies and rMuTNF-α as standard. Results are means ± s.d. *$P < 0.05$; †$P < 0.01$.

Antitumour effect of zymocel against the solid form of sarcoma 180

The antitumour effect of zymocel was examined in sarcoma 180-bearing mice. Zymocel showed significant dose-dependent activity against sarcoma 180. Intraperitoneal administration of zymocel resulted in greater activity than intravenous administration (Table 6).

Effect of administration of zymocel on leukocyte number in cyclophosphamide-induced leukopenia

Increasing the number of white blood cells by administration of biological response modifiers is an important factor in reducing the risk of catching various infectious diseases in patients suffering from immune suppression. Administration of zymocel resulted in significant dose-dependent recovery of the number of white blood cells at day 7 in comparison with the effect of saline (Fig. 1).

Effect of zymocel on in-vitro macrophage function

The direct action of zymocel on macrophage function was examined by adding zymocel to protease-peptone-elicited

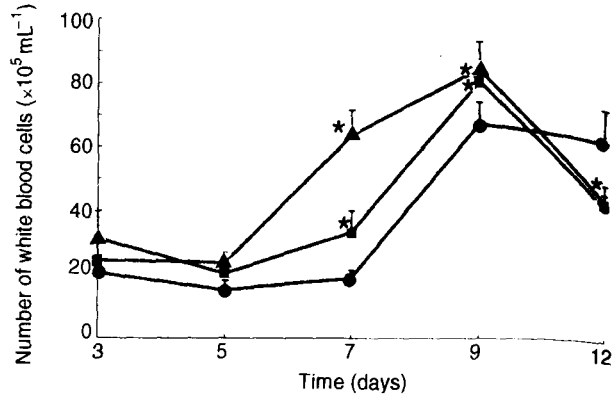

FIG. 1. Effect of zymocel on leukocyte number in cyclophosphamide-induced leukopenia. On day 0 cyclophosphamide (200 mg kg^{-1}) was administered intraperitoneally to ICR mice then zymocel was immediately administered intraperitoneally. Leukocyte number was counted on days 3, 5, 7, 9 and 12 by mixing blood with Turk's solution. ●, cyclophosphamide; ■, cyclophosphamide + zymocel (50 μg per mouse); ▲, cyclophosphamide + zymocel (250 μg per mouse). *$P < 0.001$.

macrophage or RAW264.7 macrophage-like cell line in-vitro and measuring synthesis of H_2O_2 and TNF-α. As shown in Tables 7 and 8, zymocel directly and dose-dependently activated macrophage release of H_2O_2 and TNF-α.

Discussion

Although it is well-known that infectious fungi contain various polysaccharides, such as mannans and glucans, the role and immunopharmacological activity of the polysaccharides have not yet been clearly demonstrated. We have, therefore, investigated the immunopharmacological activity of the insoluble glucan preparation zymocel and compared its activity with that of a glucan-containing particle, zymosan and with that of soluble glucans.

Administration of zymocel increased peritoneal exudate cell number and spleen weight; it also enhanced phagocytic activity, H_2O_2 production, nitric oxide production of peritoneal exudate cells, the extravascular release of Evans blue, lipopolysaccharide-triggered TNF synthesis and recovery of white blood cell number in cyclophosphamide-induced leukopenia. Administration of zymocel also showed antitumour activity against sarcoma 180 in ICR mice. These results strongly suggest that zymocel has a variety of immunopharmacological activity owing to induction of the inflammatory response and the subsequent activation of immune systems.

Table 6. Antitumour activity of particulate glucans against the solid form of sarcoma 180.

Glucan	Route	Dose (μg/mouse)	Mean ± s.d. (g)	Inhibition	CR*/n	*t*-test (to control)
Zymocel	Intravenous	50	5·1 ± 3·3	34·2	1/10	—
Zymocel	Intravenous	250	0·8 ± 2·2	89·7	7/10	$P < 0.01$
Zymocel	Intraperitoneal	50	2·5 ± 3·3	68·2	4/10	$P < 0.01$
Zymocel	Intraperitoneal	250	0·6 ± 1·5	92·9	8/10	$P < 0.01$
Zymosan	Intravenous	1000	2·3 ± 3·6	69·9	3/10	$P < 0.01$
SPG	Intraperitoneal	50	0·3 ± 0·5	96·8	8/10	$P < 0.01$
Control	—	—	7·7 ± 2·5	—		

β-Glucans were administered three times to ICR mice, 1 week after subcutaneous inoculation of sarcoma-180 carcinoma into the right groin. *Complete regression.

Table 7. Effect of zymocel on H_2O_2 production by protease peptone-elicited macrophages.

	Concn of hydrogen peroxide (nmol mL^{-1})
Zymocel 50 μg mL^{-1}	2·7 ± 0·3
+ laminarin 250 μg	0·3 ± 0·1
Zymocel 100 μg mL^{-1}	2·8 ± 0·1
+ laminarin 250 μg	1·4 ± 0·4
Zymocel 200 μg mL^{-1}	3·8 ± 0·2
+ laminarin 250 μg	1·7 ± 0·2

Protease peptone elicited macrophages were cultured with different concentrations of zymocel and H_2O_2 production was measured. Results are means ± s.d.

Table 8. Effect of zymocel on TNF-α synthesis by RAW264·7 macrophage-like cell line.

	TNF-α titre (ng mL^{-1})
Control	1·7 ± 0·2
Zymocel 20 μg mL^{-1}	4·2 ± 0·3*
Zymocel 100 μg mL^{-1}	7·7 ± 0·4*
Zymocel 500 μg mL^{-1}	9·0 ± 0·4*

RAW264·7 cells were cultured with different concentrations of zymocel and TNF-α concentration in the culture fluid was measured by ELISA using anti-TNF-α antibodies and rMuTNF-α as standard. Results are means ± s.d. *$P < 0·001$.

Comparison of the effects of zymocel with those induced by soluble glucans showed that enhancement of vascular permeability in-vivo and the triggering of H_2O_2 synthesis in-vitro were characteristic of zymocel. They were also shown by zymosan. Insoluble glucans such as zymosan activate an alternative complement pathway resulting in the production of the anaphylatoxic peptides C3a and C5a (Pillemer & Eaker 1941). These peptides could enhance vascular permeability. Compounds in the arachidonate family, leukotrienes, prostaglandins and thromboxans, could be generated by polymorphonuclear leukocytes and macrophages by zymosan (Czop & Austen 1985a). Arachidonates could affect the endothelium, and so it is suggested that released arachidonates would lead to enhanced vascular permeability. We have previously shown that soluble glucans could activate a complement pathway but the extent of the effect was significantly lower than that induced by the particulate glucans (Adachi et al 1990) and that none of the soluble glucans induced the release of arachidonates from macrophages (unpublished results). Similarly, zymocel directly triggers macrophage H_2O_2 production, but soluble glucans could not. These difference would be significantly important differences between soluble and particulate glucans.

We examined other activity in addition to the effect on vascular permeability and the triggering of H_2O_2 production. The soluble glucans were shown to enhance: phorbol myristate acetate- or insoluble glucan-induced H_2O_2 synthesis and phagocytosis by GRN (from *Grifola frondosa*), SSG, SPG, and OL-2 (from *Omphalia lapidescens*); nitric oxide production and lipopolysaccharide-triggered TNF synthesis by GRN, SSG, and OL-2; antitumour activity by GRN, SSG, and SPG; enhanced white blood cell recovery in cyclophosphamide induced leukopenia by SSG and SPG, etc. (Ohno et al 1987, 1993, 1995a, b; Suzuki et al 1989; Sakurai et al 1992). The last activities might not be different for soluble and particulate glucans and the structure-activity relationship of these activities might be different.

Czop & Austen (1985b) proposed the presence of a β-glucan-specific receptor on mononuclear phagocytes. Neither amino acid sequences nor nucleotide sequences have, however, yet been published. We have already reported that zymosan-mediated hydrogen peroxide synthesis was inhibited by soluble β-glucans (Adachi et al 1993). It was also of interest that none of the soluble glucans could produce hydrogen peroxide itself. It is generally accepted that the respiratory burst could be induced by stimuli other than β-glucan-containing particles, e.g. bacteria and opsonized particles. This suggests that particulate-glucan-mediated hydrogen peroxide synthesis might proceed, at least in part, by 'phagocytic stages' in addition to the binding to the β-glucan receptor. Such stages would require other receptors and rearrangement of the cytoskeleton. Soluble glucans might be too small to proceed by phagocytic stages. Thus, recognition of either soluble or particulate β-glucan by the β-glucan receptor, at least partly, initiates signals for a variety of immunopharmacological activity.

The results in this paper suggest that zymocel-mediated immunopharmacological activity could be expressed through various steps in-vivo. We therefore believe that the cytokine network, in addition to the direct macrophage activation, might be important in the expression of the immunopharmacological activity induced by administration of zymocel. It is, however, well-known that insoluble glucan induces the production of hydrogen peroxide from peritoneal macrophages during respiratory burst and so there is a possibility that unknown reactants are released from macrophages during the binding of zymocel, and activate the macrophages which produce inflammatory cytokines. It is, in addition, suggested that the activated macrophages could react synergistically with zymocel to induce the variety of immunopharmacological activity, i.e. the production of other cytokines and the activation of inducible nitric oxide synthetase. Considering these facts, it is suggested that the administration of zymocel induces inflammatory responses, and the subsequent immune response, via the cytokine network, especially production of interferon-γ, and that the cytokine-activated macrophages exhibit a variety of immunopharmacological activity including antitumour activity. The recognition of zymocel by activated macrophages through the β-glucan receptor might synergistically enhance the response to cytokines.

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