Signal Transduction Pathway on β -Glucans-Triggered Hydrogen Peroxide Production by Murine Peritoneal Macrophages *in Vitro*

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It has been reported that the biological activities of several β -glucans vary due to differences in their physicochemical properties. In this study we investigated the ability of β -glucans to trigger H_2O_2 production and activate signaling pathway on peritoneal macrophages. The most effective β -glucan tested on H_2O_2 production was zymocel which was a particulate preparation from the yeast cell wall. In contrast, gel-forming β -glucans which are known as immunoenhancers did not trigger the H_2O_2 production by macrophages at all. To identify the related signaling pathway for the particulate β -glucans-triggered H_2O_2 production, several inhibitors were applied. Hydrogen peroxide production triggered with phorbol myristate acetate was inhibited by pretreatment of macrophages with H-7, a protein kinase C inhibitor. However, β -glucans-triggered H_2O_2 production was not affected by H-7. The results suggested that genistein-sensitive tyrosine kinase and bromophenacyl bromide-sensitive phospholipase A_2 participated in the particulate β -glucans-triggered H_2O_2 production, although the phagocytosis of particulate β -glucans was not inhibited by either reagents. In conclusion, gel-forming (1 $\rightarrow$ 3)- β -D-glucans-induced activation was not sufficient to trigger H_2O_2 on macrophages, and pathways for particulate β -glucans-triggered H_2O_2 production were regulated differently from those for phagocytosis of β -glucans.

Key words (1 $\rightarrow$ 3)- β -D-glucan; particulate β -glucan; macrophage; hydrogen peroxide; tyrosine kinase; phospholipase A_2

β -Glucans are one of the naturally abundant materials from the cell walls of yeast, fungi and plants. These β -glucans can be classified into three types according to their physicochemical properties: 1) higher molecular weight branched (1 $\rightarrow$ 3)- β -D-glucans, composed of single- or triple-helix conformers of main glucosyl chain in water.¹⁾ The gel-forming antitumor (1 $\rightarrow$ 3)- β -D-glucans, such as grifolan (GRN; from *Grifola frondosa*), sonifilan (SPG; from *Schizophyllum commune*), and lentinan (from *Lentinus edodes*), belong to this classification.^{2–5)} 2) Lower molecular weight (<10 kDa) β -glucans or chemically modified β -glucans by substitution with a hydrophilic group into a hydroxyl group of glucosyl residues (e.g., CM-, HE-, or PC- β -glucan) have highly molecular motility and randomly coiled conformation.^{6,7)} 3) Insoluble β -glucans are typical particulates like zymosan (ZyM) and zymocel (ZyC) prepared from the yeast *Saccharomyces cerevisiae* as a carbohydrate-rich cell wall fraction^{8,9)}; the structures of this third group are assumed to have non-uniform branches and backbone units.

Reactive oxygen species (ROS), including superoxide anion (O_2^-), hydroxy radical ($OH^\bullet$), singlet oxygen (1O_2), and hydrogen peroxide (H_2O_2) are produced by phagocytes and act to kill micro-organisms.¹⁰⁾ Recently, it was also shown that ROS act as intermediators in cell activation.^{11,12)} It is known that ROS are led by the activation of NADPH oxidase which consists of membrane flavocytochrome b_{558} containing haem, flavin and NADPH-binding sites and three cytosolic components, p47-phox, p67-phox and rac 2, a ras-related GTP-binding protein.^{13,14)}

Christiansen *et al.* showed that phorbol myristate acetate (PMA)- and opsonized ZyM (OP-ZyM)-induced ROS was involved in the activation of membrane associated protein kinase C (PKC) on human neutrophils.¹⁵⁾

Dana *et al.* reported that phospholipase A_2 (PLA $_2$) activity in the signaling pathway was enhanced during activation of the NADPH oxidase complex in human neutrophils, and that PLA $_2$ is located downstream of PKC and does not activate PKC.¹⁶⁾ Moreover, it was reported that OP-ZyM-induced O_2^- generation and arachidonic acid release were regulated by pertussis toxin sensitive G-protein.¹⁷⁾ A previous report suggested that ZyM-triggered H_2O_2 production from murine peritoneal macrophages was mediated by β -glucan specific receptor.¹⁸⁾ However, the activation pathway for the β -glucan-triggered ROS production is still unclear. In this study, we investigated the ability of several β -glucans to trigger H_2O_2 production and to activate signaling pathway on peritoneal macrophages.

We found only particulate β -glucans were able to activate macrophages to produce H_2O_2 *in vitro*. The activation signals for particulate β -glucan-triggered H_2O_2 production were transduced through genistein-sensitive protein tyrosine kinase (PTK) and the PLA $_2$ inhibitor, 4-bromophenacyl bromide (pBPPB)-sensitive PLA $_2$. The herbimycin- and tyrphostin-sensitive PTK was not effective in H_2O_2 production, but PTK is important for phagocytosis of particulate β -glucans. These results suggested that PLA $_2$ and PTK activation are involved in particulate β -glucans-triggered H_2O_2 production.

MATERIALS AND METHODS

Animals Male ICR mice were purchased from Japan S.L.C. (Hamamatsu, Japan) and used at 6 to 8 weeks of age.

Reagents Lipopolysaccharide (LPS, obtained from *Escherichia coli* 0111), PMA, scopoletin, sodium orthovanadate, and horseradish peroxidase (HPO) were

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purchased from Sigma (Sigma Chemical Co., St. Louis, MO). H-7, HA1004, W-7, and W-5 were purchased from Seikagaku Kogyo Co., Ltd., Tokyo. Genistein, and tyrphostin were purchased from GIBCO-BRL Life Technologies, Grand Island, MD. Herbimycin A (herbimycin) was purchased from Wako Pure Chemical Co., Ltd., Osaka. pBPB were purchased from DOJIN, Kumamoto.

Preparation of Several β -Glucans GRN was prepared by the method described previously.¹⁹⁾ SPG was generously provided by Kaken Pharmaceutical Co., Ltd., Tokyo. Laminarin (LAM) and dextran (DEX) were purchased from Sigma Chemical Co., St. Louis, MO and Seikagaku Kogyo Co., Tokyo, respectively. Zymosan A (ZyM) and ZyC were purchased from Sigma Chemical Co., St. Louis, MO and Alpha-Beta Technology, INC., Massachusetts, respectively.

Preparation of Peritoneal Macrophages Peritoneal macrophages were isolated from ICR mice, which were injected intraperitoneally with 2 ml of proteoseptone 3 d prior to peritoneal lavage with 14 ml of Hanks' balanced salt solution (HBSS, Nissui Pharmaceutical Co., Ltd., Tokyo) containing 5 mM HEPES (Sigma Chemical Co., St. Louis, MO), 100 U/ml penicillin, 100 μ g/ml streptomycin (Meiji Seika Kaisha, Ltd., Tokyo), and 5 U/ml heparin (Wako Pure Chemical Co., Ltd., Osaka). Collected cells were washed twice with RPMI1640 and then cultured in RPMI1640 containing 1% heat inactivated fetal calf serum (FCS). The cells were allowed to adhere to a 96-well culture plate (Sumitomo Bakelite Co., Ltd., Tokyo) for 3 h at 37 °C in a 5% CO₂ incubator in 100 μ l of culture media. The culture was then washed twice with 100 μ l/well of culture media for assay to remove nonadherent cells.

H₂O₂ Assay Production of H₂O₂ was assayed continuously at 37 °C with the micro-assay using the scopoletin technique established by De la Harpe and Nathan.²⁰⁾ Briefly, peritoneal macrophages (1.25×10^5 cell/well) were washed twice with 100 μ l of HBSS (except phenol red: Nissui Pharmaceutical Co., Ltd., Tokyo). The assay mixture consisted of 30 μ M scopoletin, 1 mM NaN₃, 1 purpurogallin unit/ml HPO, 50 ng/ml PMA or 50 μ g/ml ZyM and ZyC in HBSS (except phenol red) was replaced in each well. Immediately after the addition of the assay mixture the plate was placed in a filter fluorometer (microplate reader MTP-32, Corona Electric Co., Ltd., Tokyo) and the fluorescence was recorded with an excitation wavelength of 365 nm and an emission wavelength of 450 nm. After incubation for 2 h at 37 °C, the fluorescence in each well was again recorded using the fluorometer. The amount of H₂O₂ produced by macrophages was calculated from the fluorescence reduction using H₂O₂ solution as standard.

Assay of Binding and Phagocytosis by Macrophages Phagocytic activity was assayed by the method described previously.²¹⁾ Briefly, peritoneal macrophages (1×10^5 cells/well) washed twice with 100 μ l of RPMI1640 were incubated with 1×10^7 of Fluorescein-conjugated zymosan (Fl-ZyM: prepared from zymosan A) in RPMI1640 containing 10% FCS in 96-well flat-bottomed tissue culture plates for 30 min at 37 °C. The macrophages were then washed 3 times with warmed HBSS (except phenol

red) to remove any nonphagocytosed Fl-ZyM, and solubilized by the addition of 100 μ l of 50 mM of sodium cholate. The fluorescence intensity was measured with a microplate reader with an excitation wavelength of 490 nm and an emission wavelength of 530 nm. The phagocytic activity was calculated using the following formula: phagocytic activity (%) = (fluorescein intensity of phagocytosed Fl-ZyM by reagent treated peritoneal macrophages/fluorescein intensity of phagocytosed Fl-ZyM by control peritoneal macrophages) $\times$ 100.

RESULTS

Effect of β -Glucans on H₂O₂ Production by Macrophages Several physicochemically different (1 $\rightarrow$ 3)- β -D-glucans, such as gel-forming (1 $\rightarrow$ 3)- β -D-glucans, randomly coiled β -glucans, and particulate β -glucans, were tested for their ability to trigger H₂O₂ production by macrophages. Macrophages incubated with gel-forming (1 $\rightarrow$ 3)- β -D-glucans, including GRN and SPG, were not significantly activated on H₂O₂ production (Table 1). Only particulate β -glucans, ZyM and ZyC, could trigger H₂O₂ production. This production by particulate β -glucans showed a dose dependent manner, and ZyC was more effective than ZyM at the lowest concentration (25 μ g/ml, $p < 0.05$).

Effect of PKC Inhibitor and Calmodulin Antagonist on Particulate β -Glucans-Triggered H₂O₂ Production by Macrophages To investigate the activation pathway of H₂O₂ production by particulate β -glucans, macrophages were incubated with the β -glucans in the presence of PKC inhibitor, H-7. Macrophages were pretreated with various doses of H-7 for 10 min at 37 °C, then cells were stimulated with either 50 μ g/ml of particulate β -glucans or 50 ng/ml of PMA in the presence or absence of inhibitors for 2 h at 37 °C. As shown in Table 2, PMA-triggered H₂O₂ production was almost suppressed by H-7. This inhibitory effect was dependent on the concentration, and control reagent HA1004 which is a weaker inhibitor

Table 1. Effect of β -Glucans on H₂O₂ Production by Macrophages^{a)}

Sample	Dose (μ g/ml)	H ₂ O ₂ concentration ^{b)} (μ M, mean $\pm$ S.D.)
Control		0.00 $\pm$ 0.00
LPS	0.5	0.42 $\pm$ 0.15
GRN	100	0.00 $\pm$ 0.00
	250	0.00 $\pm$ 0.00
SPG	100	0.00 $\pm$ 0.00
	250	0.00 $\pm$ 0.00
LAM	100	0.00 $\pm$ 0.00
	250	0.00 $\pm$ 0.00
ZyM	25	7.98 $\pm$ 0.00
	50	11.26 $\pm$ 0.41
	75	12.21 $\pm$ 0.29
	100	12.63 $\pm$ 0.13
ZyC	25	9.52 $\pm$ 0.48
	50	11.30 $\pm$ 0.18
	75	12.31 $\pm$ 0.48
	100	12.28 $\pm$ 0.04

a) Macrophages were stimulated with or without LPS and several β -glucans for 2 h at 37 °C in a humidified CO₂ incubator. b) Produced H₂O₂ was measured by reduction of fluorescence intensity of scopoletin. The data are the mean of triplicate stimulations in a single experiment.

of PKC than H-7 did not suppress the production significantly. Particulate β -glucans-triggered H_2O_2 production, in contrast, was not affected by H-7. However, calmodulin antagonist W-7 inhibited the H_2O_2 production triggered by particulate β -glucans, while the negative control reagent, W-5, did not (data not shown).

Effect of PTK Inhibitors on Particulate β -Glucans-Triggered H_2O_2 Production by Macrophages To determine whether PTK is involved in the signaling pathway of β -glucans-triggered H_2O_2 production by macrophages, two specific PTK inhibitors, herbimycin and genistein, were applied (Table 3). A number of studies have shown that these inhibitors are effective against most tyrosine kinases, but do not inhibit cyclic nucleotide-dependent protein kinases (PKA, PKG), PKC, phosphorylase kinase, or phospholipase C.^{22,23} Macrophages were incubated with the inhibitors for 1 h at 37 °C prior to stimulation with 50 μ g/ml of particulate β -glucans or 50 ng/ml of PMA for 2 h at 37 °C. Particulate β -glucans-triggered H_2O_2 production was suppressed with genistein in a dose dependent manner, while herbimycin did not inhibit the production. PMA-triggered H_2O_2 production was not affected by genistein, and was only inhibited by herbimycin at above 0.5 μ g/ml (data not shown).

We also investigated the effect of the protein tyrosine phosphatase (PTPase) inhibitor, vanadate, on particulate β -glucans-triggered H_2O_2 production. Other investigators reported that vanadate at micromolar concentrations

was effective and rather selective as an inhibitor of PTPase.^{24,25} Macrophages were stimulated with 50 μ g/ml of particulate β -glucans in the presence or absence of vanadate for 2 h at 37 °C. The H_2O_2 production triggered with particulate β -glucans was not changed significantly in either preparation (data not shown).

Effect of PLA₂ Inhibitor on ZyM- and PMA-Induced H_2O_2 Production by Macrophages Arachidonic acid reportedly was released from ZyM-activated macrophages, and the arachidonic acid itself stimulated superoxide production in intact cells.¹⁶ The release of arachidonic acid and the involvement of PLA₂ are suggested to have important roles for the NADPH oxidase activity in monocytes stimulated with opsonized ZyM.²⁶ First, in order to investigate whether PLA₂ is involved in the H_2O_2 production by macrophages induced with particulate β -glucans, a specific inhibitor of PLA₂, pBPB, was used (Fig. 1). Macrophages were incubated with pBPB for 5 min at 37 °C, and subsequently stimulated with 50 μ g/ml of ZyM or 50 ng/ml of PMA for 2 h at 37 °C. Both ZyM- and PMA-induced H_2O_2 production were inhibited by pBPB in a dose dependent manner, and maximum inhibition was observed at more than 6.7 μ M.

Effect of Various Inhibitors on Interaction between FI-ZyM and Macrophages We examined the effect of various inhibitors for the signal transduction pathways on macrophage interaction with ZyM believing that there might be a relation between these pathways and ZyM-induced bioactivity, and that a linkage regulated mechanism functioned in macrophages activation by ZyM. Macrophages were incubated with the inhibitors at 37 °C before stimulation with FI-ZyM 1×10^7 particulates/ml for 0.5 or 1 h at 37 °C or 4 °C, respectively. As shown in Table 4, the phagocytosis of FI-ZyM by macrophages was not affected by PKC inhibitor: H-7, PTK inhibitor: genistein, PTPase inhibitor: vanadate, or PLA₂ inhibitor: pBPB. However, PTK inhibitors: tyrphostin and herbimycin inhibited the FI-ZyM phagocytosis about 15% (10 μ g/ml) and 20% (0.5 μ g/ml), respectively, and Ca²⁺ chelator, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) and calmodulin antagonist, W-7 were the most effective in inhibition. The binding of FI-ZyM to macrophages incubated for 1 h at 4 °C was not affected by pretreatment with inhibitors (data not shown).

Table 2. Effect of PKC Inhibitors on Particulate β -Glucans-Triggered H_2O_2 Production by Macrophages^{a)}

Inhibitor	Dose (μ M)	H_2O_2 concentration (μ M, mean $\pm$ S.D.) ^{b,c)}		
		PMA 50 ng/ml	ZyC 25 μ g/ml	ZyC 50 μ g/ml
Control		7.03 $\pm$ 0.21	7.70 $\pm$ 1.34	10.41 $\pm$ 1.84
H-7	20	2.06 $\pm$ 0.37**	7.35 $\pm$ 1.05	10.54 $\pm$ 0.41
	30	1.45 $\pm$ 0.24**	7.25 $\pm$ 0.21	10.31 $\pm$ 0.35
	40	n.d.	7.02 $\pm$ 0.23	9.51 $\pm$ 0.59
HA1004	20	6.20 $\pm$ 0.15	n.d.	n.d.
	30	5.95 $\pm$ 0.16	7.94 $\pm$ 0.08	10.65 $\pm$ 0.23
	40	n.d.	7.49 $\pm$ 0.41	9.91 $\pm$ 0.09

a) Macrophages were pretreated with or without increasing concentrations of H-7 and HA1004 in HBSS for 10 min at 37 °C. Cells were then stimulated with PMA or ZyC for 2 h at 37 °C in a humidified CO₂ incubator. b) Produced H_2O_2 was measured by reduction of fluorescence intensity of scopoletin. The data are the mean of triplicate stimulations in a single experiment. c) n.d., not determined. ** $p < 0.01$.

Table 3. Effect of PTK Inhibitors on Particulate β -Glucans-Triggered H_2O_2 Production by Macrophages^{a)}

Sample	H_2O_2 concentration (μ M, mean $\pm$ S.D.) ^{b,c)}				
	Vehicle	Genistein (μ g/ml)		Herbimycin (μ g/ml)	
		1	10	0.1	0.2
Control	0.21 $\pm$ 0.23	n.d.	n.d.	n.d.	n.d.
PMA 50 ng/ml	7.83 $\pm$ 0.05	7.88 $\pm$ 0.23	7.48 $\pm$ 0.00	7.32 $\pm$ 0.02	n.d.
ZyM 50 μ g/ml	6.67 $\pm$ 0.25	5.58 $\pm$ 0.48	2.84 $\pm$ 0.31**	6.43 $\pm$ 0.64	6.47 $\pm$ 0.67
ZyC 50 μ g/ml	9.33 $\pm$ 0.24	6.77 $\pm$ 0.19**	3.34 $\pm$ 0.45**	9.38 $\pm$ 0.15	9.55 $\pm$ 0.70

a) Macrophages were pretreated with or without increasing concentration of genistein, herbimycin or vehicle (dimethyl sulfoxide, DMSO) in HBSS for 60 min at 37 °C. Cells were then stimulated with PMA, ZyM or ZyC for 2 h at 37 °C in a humidified CO₂ incubator. b) Produced H_2O_2 was measured by reduction of fluorescence intensity of scopoletin. The data are the mean of triplicate stimulation in a single experiment. c) n.d., not determined. ** $p < 0.01$.

Fig. 1. Effect of PLA₂ Inhibitor on PMA- and Particulate β -Glucan-Triggered H₂O₂ Production by Macrophages

Macrophages were pretreated with or without increasing concentration of pBPB in HBSS for 5 min at 37 °C. Cells were then stimulated with 50 ng/ml of PMA ($\blacktriangle$), 50 μ g/ml of ZyM ($\blacksquare$) or not ($\circ$) for 2 h at 37 °C in a humidified CO₂ incubator. Produced H₂O₂ was measured as described in Materials and Methods. * $p < 0.05$, ** $p < 0.01$ refer to comparisons between macrophage treated inhibitors and control.

Table 4. Effect of Several Inhibitors on FI-ZyM Phagocytosis by Macrophages^{a)}

Inhibitors			FI-ZyM phagocytosis (% of control, mean $\pm$ S.D.)
H-7	(μM)	20	106.06 $\pm$ 3.93
		30	105.06 $\pm$ 1.64
		40	102.07 $\pm$ 3.70
HA1004	(μM)	20	96.58 $\pm$ 2.31
		30	86.06 $\pm$ 10.03
		40	90.53 $\pm$ 5.16
W-7	(μM)	20	93.14 $\pm$ 15.84
		30	47.60 $\pm$ 5.11**
		40	39.53 $\pm$ 5.78**
W-5	(μM)	20	98.20 $\pm$ 7.49
		30	109.53 $\pm$ 21.83
		40	106.20 $\pm$ 8.20
BAPTA	(μM)	10	68.43 $\pm$ 6.51*
		20	24.12 $\pm$ 13.14*
		30	25.54 $\pm$ 4.87**
Genistein	$(\mu\text{g/ml})$	5	101.48 $\pm$ 1.79
		10	100.59 $\pm$ 4.29
		20	94.91 $\pm$ 1.25
Tyrphostin	$(\mu\text{g/ml})$	1	99.77 $\pm$ 3.51
		5	92.45 $\pm$ 1.30*
		10	85.15 $\pm$ 2.10**
Herbimycin	$(\mu\text{g/ml})$	0.2	79.87 $\pm$ 1.64**
		0.5	71.67 $\pm$ 2.33**
pBPB	(μM)	6.7	103.52 $\pm$ 1.44
		10.0	107.33 $\pm$ 4.43
		15.0	112.03 $\pm$ 6.07

a) Macrophages were prepared with or without increasing concentrations of H-7, HA1004, W-7 and W-5 in RPMI1640/10% FCS for 10 min at 37 °C. Macrophages were pretreated with or without increasing concentrations of BAPTA-AM, genistein, tyrphostin, herbimycin or vehicle (DMSO) in RPMI1640/10% FCS for 60 min at 37 °C. Macrophages were pretreated with or without increasing concentration of pBPB in RPMI1640/10% FCS for 5 min at 37 °C. Cells were then cultured with FI-ZyM 1.0×10^7 /ml in RPMI1640/10% FCS for 30 min at 37 °C in a humidified 5% CO₂ incubator. b) Amount of FI-ZyM in cell lysate of macrophages was measured as described in Materials and Methods. * $p < 0.05$, ** $p < 0.01$.

DISCUSSION

We investigated the signaling pathway for β -glucan triggered H₂O₂ production in peritoneal macrophages. First, the effects of several β -glucans were examined to determine their structure-activity relationships to H₂O₂ production *in vitro*. The soluble low-molecular weight β -glucan, LAM and gel-forming (1 $\rightarrow$ 3)- β -D-glucans, GRN and SPG, did not induce H₂O₂ production, while particulate β -glucans, ZyM and ZyC, did (Table 1). However, murine peritoneal macrophages elicited by administration of GRN and SPG showed enhanced H₂O₂ production triggered by ZyM (data not shown). The H₂O₂ production can be induced by two sequential signals: a priming signal and a triggering signal. Our data indicated the gel-forming (1 $\rightarrow$ 3)- β -D-glucans did not generate the triggering signal in macrophages, although that complement receptor, CR3 (CD11b/CD18) reportedly participated in ZyM-induced biological activities and phagocytosis of ZyM particulates.²⁷⁻²⁹⁾ The present study showed possibility that there might be different recognition mechanisms between particulate β -glucans and gel-forming (1 $\rightarrow$ 3)- β -D-glucans. In our previous study using various kinds of β -glucans, branching ratio and molecular weight of gel-forming (1 $\rightarrow$ 3)- β -D-glucans were important for induction of cytokine from macrophages *in vitro*.³⁰⁾ Therefore, multiple recognition mechanisms for the several kinds of β -glucans might affect biological activities in macrophage. Poutsika *et al.* reported that the ZyC-derived soluble hydrolysis product, betafectin, did not induce production of interleukin-1 receptor antagonist (IL-1ra) by human mononuclear cells, although immobilized betafectin on culture plate did.³¹⁾ In our experiment, however, immobilized GRN on culture plate had no effect on H₂O₂ production (data not shown). The activation of cytokine production and the triggering of H₂O₂ production by β -glucans were believed to be regulated by quite different pathways.

To determine intra-cellular signaling in H₂O₂ production triggered by particulate β -glucans, cells were treated with several inhibitors; at the concentrations tested, however the inhibitors had no effect on cell-viability. We examined the effect of a PKC inhibitor that inhibits ZyM-triggered cytokine production by human monocytes²⁹⁾ on particulate β -glucans-triggered H₂O₂ production. Lower concentration of H-7 (10 nM) suppressed PMA-triggered H₂O₂ production (50%), but higher concentration (40 nM) did not affect ZyM-triggered production (Table 2). Levy *et al.* showed previously that treatment with H-7 and staurosporine, which are potent inhibitors of PKC, suppressed p47 phosphorylation, but did not affect NADPH oxidase activity in opsonized ZyM (OP-ZyM)-activated neutrophils.³²⁾ Our data agreed with their report showing no effect of H-7 on NADPH oxidase activation pathway. In addition, Dusi *et al.* showed that the translocation of the cytosolic factors is independent of PKC activity and is linked to transmembrane signaling involving Ca²⁺ transients and production of lipidic second messenger.³³⁾ Taken together, these appeared to suggest that particulate β -glucans-triggered H₂O₂ production did not require signaling pathway for

ZyM-induced cytokine production or PMA-activated PKC pathway. Particulate β -glucans-triggered H_2O_2 production was inhibited by genistein-sensitive PTK, which was not affected by herbimycin (Table 3), although PMA-triggered H_2O_2 production was not affected by PTK inhibitors. Interestingly, this observation did not correlate with phagocytosis of FI-ZyM, which was suppressed by herbimycin ($0.5 \mu\text{g/ml}$, $71.67 \pm 2.33\%$), and by the PTK specific inhibitor, tyrphostin ($10 \mu\text{g/ml}$, $85.15 \pm 2.10\%$). Magae *et al.* reported that genistein inhibited phagocytosis of yeast by thioglycollate-elicited macrophages, and the phagocytosis process was regulated by PTK dependent pathway rather than PKC.³⁴⁾ It was reported that genistein-inhibitable tyrosine phosphorylation was required for Fc-receptor-mediated phagocytosis of murine macrophages.³⁵⁾ Our results did not agree with their data, because the concentration of genistein used in our study was lower than Magae's report (Table 4), although our concentration was enough to inhibit H_2O_2 production significantly. Phagocytosis and recognition of FI-ZyM was not affected by H-7 treatment (Table 4). It was reported, however, that staurosporine inhibited CR3-mediated phagocytosis of both iC3b-opsonized sheep E and insoluble β -glucan particulates by human neutrophils.³⁶⁾ Thus our results suggested that triggering of H_2O_2 production and phagocytosis of particulate β -glucans were not regulated by a common signaling pathway. Hazeki *et al.* reported that phagocytosis and the other cellular responses are not always caused by the same mechanism, although these immuno-inflammatory responses might be functionally related to each other.¹⁷⁾ It was then suggested that genistein-sensitive PTK might be involved in particulate β -glucans-triggered H_2O_2 production from peritoneal macrophages, and that inhibition of H_2O_2 production by calmodulin antagonist and Ca^{2+} chelator would correlate with phagocytosis by macrophages.

Reports of other studies stated that ZyM could not only induce respiratory burst in phagocyte, but could also increase PLA_2 activity and arachidonic acid release.^{37,38)} Furthermore, Tarsi-Tsuk and Levy demonstrated that arachidonic acid and PLA_2 are involved in the transduction pathway stimulating the NADPH oxidase activity in peripheral blood monocytes by lipoteichoic acid and OP-ZyM.²⁶⁾ Also arachidonic acid itself stimulates superoxide production in intact cells, cell-free assay and electroporabilized cells.³⁹⁻⁴¹⁾ Badway *et al.* reported that NADPH oxidase might be activated by a PLA_2 -mediated pathway without involvement of p47 phosphorylation.⁴²⁾ The location of PLA_2 activity in the signal-transduction pathway leading to oxidase activation has not yet been defined. However, the present study demonstrated that PLA_2 activity might be a co-requirement in the H_2O_2 production triggered with particulate β -glucans. Other studies showed that ZyM-activated arachidonic acid release and O_2^- generation were inhibited by pertussis toxin (PT) treatment.¹⁷⁾ It was suggested that PLA_2 activity is regulated by the PT sensitive G protein, G_i . Our present data suggested that particulate β -glucans possess activities and that the signaling pathway might closely relate to PLA_2 activation. Therefore, it

seemed that the pathway for β -glucan-triggered H_2O_2 production was associated with G-proteins, although further investigation, especially upstream on the pathway, remains to be done.

In conclusion, the activation signals for particulate β -glucan-triggered H_2O_2 production were transduced through genistein-sensitive PTK and pBPB-sensitive PLA_2 . Herbimycin- and tyrphostin-sensitive PTK was not effective in the H_2O_2 production, and is also important for phagocytosis of particulate β -glucans. These results imply there are multiple recognition units of β -glucans which are responsible for the various activities induced with β -glucans.

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