

Original Article

Comparative study on modeccin- and phytohemagglutinin (PHA)-induced secretion of cytokines and nitric oxide (NO) in RAW264.7 cells

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The effects of cytotoxic lectins, modeccin and phytohemagglutinin (PHA) on mouse macrophage cell line RAW264.7 was studied by detecting the induction of inflammatory mediators. Results showed that modeccin induced the release of tumor necrosis factor- α (TNF- α) from RAW264.7 cells with a bell-shape concentration-dependent profile. PHA that showed no significant cytotoxicity on RAW264.7 cells up to 100,000 ng/ml induced much higher level of TNF- α than modeccin. PHA simultaneously induced the secretion of granulocyte colony stimulation factor (G-CSF) from RAW264.7 cells with even much higher level than that of TNF- α , whereas modeccin did not. Furthermore, PHA induced the secretion of nitric oxide (NO) in RAW264.7 cells, while no significant level of NO was detected in the modeccin-treated cells. NH₄Cl (a lysomotropic agent) and cycloheximide (a ribosome inhibitor) strongly inhibited modeccin-induced TNF- α secretion, but no significant inhibitory effects of these reagents on the PHA-induced TNF- α secretion were observed. Contrary to modeccin-induced TNF- α secretion, even slightly increased TNF- α secretion was observed in PHA-treated cells in the presence of 10 mM NH₄Cl. In addition, the inhibition profiles of modeccin-induced TNF- α secretion by various kinase inhibitors were different from those of PHA. These results suggested that the action mode of modeccin to stimulate RAW264.7 cells leading to the secretion of inflammatory molecules, including TNF- α , is distinct from that of PHA. On the other hand, significantly increased translocation of activator protein-1 (AP-1), a crucial transcription factor involved in expression of inflammatory molecules, into nucleus was observed in RAW264.7 cells treated with PHA and modeccin.

Keywords modeccin; PHA; TNF- α ; G-CSF; nitric oxide

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Introduction

Lectins are proteins or glycoproteins that are capable of specific recognition and binding to carbohydrate moieties of glycoproteins or glycolipids. Thus, lectins are tools generally used in studies of cell surface carbohydrate structures and oligosaccharides structures of glycoconjugates [1]. Many lectins possess various biological activities *in vitro* and *in vivo*. For instance, some lectins bind to specific carbohydrate receptors of cells, which can activate the receptors, and thereby induce intracellular signaling cascades leading to alterations in various cellular processes. It also has been reported that some lectins bind to the cells involving in the innate immune system, which can result in cytokine secretion [2,3].

Modeccin is a toxic lectin existed in the roots of the South-African plant, *Modecca digitata*. This toxic lectin contains two structurally and functionally different subunits covalently connected by a disulfide bond. One subunit, the A-chain, has enzymatic property to inactivate eukaryotic ribosomes, while the other, the B-chain, binds cell surface through interaction with *N*-acetylgalactosamine- or galactose-containing carbohydrates. B-chain binding causes the toxin to be incorporated into eukaryotic cells, probably by endocytosis, and may assist the transfer of A-chain into cytoplasm. Similar to modeccin, several plant protein toxins, such as ricin and abrin, have common structural features and are classified as type 2 ribosome-inactivating proteins (RIPs). Interestingly, it has been demonstrated that the A-chains of these type 2 RIPs are commonly *N*-glycosidases which specifically cleave the *N*-glycosidic bond of a single adenine residue in the 28S ribosomal RNA (rRNA), and eventually inhibit cellular protein synthesis [4]. Despite the structural and functional similarity among the type 2 RIPs, the members of type 2 RIPs seem to have their own specific intoxication mechanism. For instance, it has been reported that NH₄Cl, which elevates the intravesicular pH, inhibits

the cytotoxicity of modeccin, but enhances the cytotoxicity of ricin, suggesting that modeccin requires NH_4Cl -sensitive specific process for the intoxication [5]. Recent studies have demonstrated that type 2 RIPs induce apoptotic cell death *in vitro* [6–9]. Furthermore, it has been reported that modeccin induces the release of $\text{TNF-}\alpha$ and interleukin (IL)-8 from colon epithelial cell line (Caco-2) and macrophages, respectively, although the underlying mechanism is still controversial [10,11].

Phytohemagglutinin (PHA) is a homotetrameric protein with a molecular weight of 120 kDa with sugar specificity for *N*-acetylgalactosamine and galactose similar to modeccin [1,2]. PHA is also known to be cytotoxic on certain cell types [1,12], and is shown to activate T cells through binding to the carbohydrate portion of the T-cell receptor [13–15].

Macrophages are known to produce multiple inflammatory molecules such as nitric oxide (NO), $\text{TNF-}\alpha$, IL-1, IL-12, interferon- γ and chemokines in response to the stimulation of some lectins [16–18]. However, how lectin binding can initiate the intracellular signaling pathways leading to the eventual secretion of multiple biological mediators is still unclear.

Although modeccin and PHA have similar sugar specificity and both are known to be cytotoxic, there are different structural and functional features between these lectins as described above. To gain insight into the action mechanisms of these lectins on macrophages, we conducted a comparative study between modeccin and PHA by detecting the induction of inflammatory mediators' secretion in mouse macrophage cell line RAW264.7. The results obtained in this study suggested that the action mode of modeccin to stimulate RAW264.7 cells could lead to $\text{TNF-}\alpha$ secretion, which was distinct from the action mode of PHA that could induce G-CSF secretion, NO production, as well as $\text{TNF-}\alpha$ secretion. Different responses to various inhibitors were also observed between modeccin- and PHA-induced $\text{TNF-}\alpha$ secretions in RAW264.7 cells.

Materials and Methods

Materials

PHA was purchased from Sigma Chemical Co. (St. Louis, USA). Modeccin was obtained from Inland Laboratories (Austin, USA). *N*-nitro-L-arginine methyl ester (L-NAME) was purchased from Dojindo Laboratories (Kumamoto, Japan). PD98059, SB202190 and SP600125, which are specific inhibitors for extracellular-regulated kinase (ERK), p38 mitogen-activated protein (MAP) kinase and c-jun NH_2 -terminal kinase (JNK), were obtained from Calbiochem (La Jolla, USA). Piceatannol (protein tyrosine kinase inhibitor) was purchased from Sigma Chemical Co. The activator protein 1 (AP-1) consensus IRDye[®] 700

Infrared dye-labeled oligonucleotide probe was purchased from LI-COR Bioscience (Lincoln, USA). Other chemicals were of the highest grade commercially available.

Cell culture

RAW264.7 (mouse macrophage) cells were obtained from the American Type Culture Collection (Manassas, USA), and cultured in CO_2 (5%) at 37°C in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 $\mu\text{g/ml}$).

Measurement of cytotoxicity

The cytotoxic effects of lectins on RAW264.7 cells were measured by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. In brief, adherent RAW264.7 cells (3×10^4 cells per well in 96-well plates) were cultured with varying final concentrations of modeccin (0–100 ng/ml) or PHA (0–100,000 ng/ml) in the growth medium for 24 h, and then incubated with MTT for 0.5 h. After aspiration of the medium, dimethylsulfoxide was added to dissolve the MTT formazan reaction product and then the optical density was measured at 570 nm using a multi-well scanning spectrophotometer (MPR-A4i; TOSOH Co., Ltd., Tokyo, Japan). The cytotoxic potentials of three MAP kinase inhibitors (0–100 μM) and piceatannol (0–100 μM) on RAW264.7 cells were also examined by the same way.

Detection of cytokines in the supernatants of lectin-treated RAW264.7 cells

The levels of $\text{TNF-}\alpha$ and G-CSF in culture supernatants following each lectin treatment were measured by sandwich enzyme-linked immunosorbent assay (ELISA) with two antibodies to two different epitopes on each cytokine molecule by similar methods as described previously [19,20]. The ELISA procedure was performed according to the manufacturer's protocol. The cytokine concentrations were estimated from a reference to a standard curve for serial 2-fold dilution of murine recombinant cytokines. When the effects of various monosaccharides (10 or 100 mM), NH_4Cl (1 or 10 mM), cycloheximide (100 or 1000 ng/ml), three different MAP kinase inhibitors (15 μM) or piceatannol (100 μM) on the $\text{TNF-}\alpha$ levels induced by the lectins were examined, the adherent cells (3×10^4 cells per well in 96-well plates) were pre-incubated with each reagent for 1 h at 37°C in the growth medium, and then modeccin (final concentration 3 ng/ml) or PHA (final concentration 100,000 ng/ml) was added. Twenty-four hours later, the supernatant was withdrawn from each well, and subjected to the measurement of $\text{TNF-}\alpha$ level by sandwich ELISA method as described above.

Nitrite assay for the estimation of NO production

Nitrite, a stable reaction product of NO with molecular oxygen, in the supernatants from each lectin-treated RAW264.7 cells, was determined by the method based on Griess assay as described previously [21]. Briefly, Griess reagent (100 μ l, 3 mM sulfanilic acid, 30 μ M *N*-1-naphthyl-ethylenediamine dihydrochloride, 25% glacial acetic acid) was added to 50 μ l of each cultured supernatant. After 20 min of incubation at room temperature, the optical density was measured at 540 nm using a multiwell scanning spectrophotometer. Calibration curve was made with a known concentration of NaNO₂ standard solution.

Reverse transcription–polymerase chain reaction

RAW264.7 cells (5×10^5 cells per well in 24-well plates) were treated with modeccin (final concentration 3 ng/ml) or PHA (final concentration 100,000 ng/ml) for 12 h. The untreated cells were used as a control. After 12 h, total RNA of each treated cells was isolated using Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan) and the levels of iNOS mRNA were measured by semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR). In brief, total RNA (2.5 μ g) was reverse transcribed with an oligo dT primer in a 10- μ l reaction volume using PrimeScript[®] 1st strand cDNA Synthesis Kit (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's instruction. PCR was performed with one cycle for 70 s at 95°C, 25 cycles for 55 s at 93°C, 45 s at 61°C, 40 s at 72°C and one cycle for 100 s at 72°C, in a 25- μ l reaction mixture containing 12.5 μ l of GoTag Green Master Mix (Promega, Madison, WI, USA), 0.5 μ l of forward and reverse *iNOS* primers (1 μ M each) or β -actin primers (10 pM each), 0.5 μ l of first-strand cDNA and 11 μ l of nuclease-free water. The primer sequences were 5'-CAACCAGTATTA TGGCTCCT-3' (forward) and 5'-GTGACAGCCCGGTC TTTCCA-3' (reverse) for mouse *iNOS* and 5'-GGAGAAG ATCTGGCACCACACC-3' (forward) and 5'-CCTGCTTGC TGATCCACATCTGCTGG-3' (reverse) for mouse β -actin. The β -actin primer was used as an internal control. The PCR products were analyzed in 2% agarose gel electrophoresis containing 0.1 μ g/ml of ethidium bromide, and the amplified products (231 bp for *iNOS*, 840 bp for β -actin) were observed by a Light capture (ATTO Co., Tokyo, Japan).

Electrophoretic mobility shift assay

RAW264.7 cells (2×10^6 cells per well in 6-well plates) were treated with modeccin (final concentration 3 ng/ml) or PHA (final concentration 100,000 ng/ml) for 5 h. The untreated cells were used as a control. After 5 h, the nuclear proteins were extracted from the cells as previously described in detail [22]. Electrophoretic mobility shift assay (EMSA) were performed with Odyssey[®] IRD[®] 700 infrared dye-labeled double-stranded oligonucleotides

coupled with the EMSA buffer kit (LI-COR Bioscience) according to manufacturer's instructions. Briefly, 5 μ g of nuclear extract was incubated with 1 μ l of IRD[®] 700 Infrared dye-labeled double-stranded oligonucleotides, 2 μ l of 10 \times binding buffer, 2.5 mM DTT, 0.25% Tween-20 and 1 μ g of poly (dl-dC) in a total volume of 20 μ l for 30 min at room temperature in dark. Samples were separated on a 4% polyacrylamide gel in 0.25 \times Tris–borate–EDTA running buffer for 45 min at 80 V. The gel was scanned by direct infrared fluorescence detection on the Odyssey[®] Imaging System (LI-COR Bioscience). The oligonucleotide's sequences of the double-stranded DNA probes labeled with AP-1 IRDye[®] 700 infrared dye were as follows: 5'-CGCTTGATGACTCAGCCGGAA-3' and 3'-GCGAACTACTGAGTCGGCCTT-5'.

Statistical analysis

All the experiments were repeated at least three times. The results were expressed as a mean \pm SD, and the data were analyzed using one-way analysis of variance (ANOVA) followed by a Dunnett's test to determine any significant differences. This statistical analysis was conducted with the Statistical Package for the Social Sciences (SPSS) version 16.0 (SPSS, Inc., Chicago, USA). A *P*-value of <0.05 was considered statistically significant.

Results

Cytotoxic effects of modeccin and PHA on RAW264.7 cells

Figure 1 shows typical dose-dependent curves of modeccin and PHA. Modeccin showed highly cytotoxic effect on RAW264.7 cells. Based on these results, the IC₅₀ value of modeccin was estimated to be 2.0–4.0 ng/ml. PHA showed no significant cytotoxicity up to 100,000 ng/ml on this cell line.

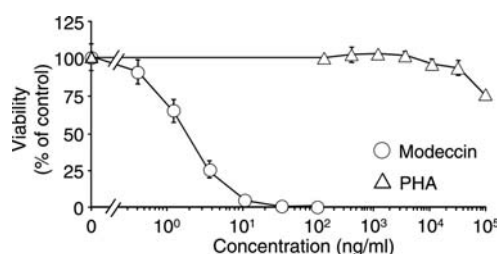


Figure 1 Cytotoxic effects of modeccin and PHA on RAW264.7 cells. Adherent RAW264.7 cells (3×10^4 cells per well in 96-well plates) were incubated with the indicated concentrations of modeccin or PHA in DMEM containing 10% of FBS at 37°C. After 24 h, the viabilities of the cells were examined by MTT assay as described in the text. Data were presented as mean \pm SD, *n* = 3.

Cytokine-inducing activities of modeccin and PHA on RAW264.7 cells

Modeccin induced the release of TNF- α from RAW264.7 cells in a concentration-dependent manner but with bell-shape profile [Fig. 2(A)]. This may be due to the potent cytotoxicity at higher concentrations than a certain critical point (Fig. 1). The significant release of TNF- α was attained with the modeccin concentration of 2.5–10.0 ng/ml. On the other hand, PHA induced much higher level of TNF- α compared with modeccin, and the increased level of TNF- α along with the increasing lectin concentration were induced [Fig. 2(B)]. Since our previous study on C-type lectin, CEL-I, demonstrated that granulocyte-colony stimulating factor (G-CSF) was secreted with much higher level than TNF- α from CEL-I-stimulated RAW264.7 cells [23], we also examined the level of G-CSF in the culture medium of modeccin- or PHA-treated RAW264.7 cells. As shown in Fig. 2(B), in addition to TNF- α , G-CSF was also detected in the culture supernatant of PHA-treated RAW264.7 cells, and the level of G-CSF was even much higher than that of TNF- α , whereas no significant level of G-CSF was detected in the modeccin-treated cells [Fig. 2(A)].

NO levels in the culture supernatant of modeccin- and PHA-treated RAW264.7 cells

As shown in Fig. 3, PHA induced NO production in RAW264.7 cells in a concentration-dependent manner, while no significant levels of NO were detected in the modeccin-treated cells at the concentration range tested. To analyze the source of NO in PHA-treated RAW264.7 cells, we used L-NAME, a specific inhibitor of NO synthase (NOS). In agreement with the previous report that inducible NOS (iNOS) is a main enzyme responsible for NO production in activated RAW264.7 cells [21], the production of NO by the PHA-treated RAW264.7 was inhibited by L-NAME (10 mM) (Fig. 3). RT-PCR analysis confirmed the expression of

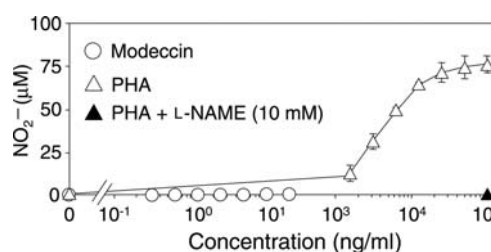


Figure 3 NO levels in modeccin- and PHA-treated RAW264.7 cells Adherent RAW264.7 cells (3×10^4 cells per well in 96-well plates) were incubated with the indicated concentrations of modeccin or PHA in DMEM containing 10% of FBS at 37°C. After 24 h, the supernatant was withdrawn from each well, and subjected to the measurement of NO levels as described in the text. Closed triangle indicates the NO level induced by 100,000 ng/ml PHA in the presence of 10 mM L-NAME, an iNOS inhibitor. Data were presented as mean \pm SD, $n = 3$.

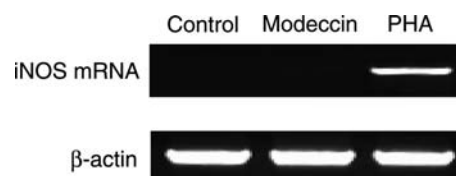


Figure 4 iNOS mRNA levels in modeccin- and PHA-treated RAW264.7 cells RAW264.7 cells (5×10^5 cells per well in 24-well plates) were treated with modeccin (final 3 ng/ml) or PHA (final 100,000 ng/ml) in DMEM containing 10% of FBS at 37°C. The untreated cells were used as a control. Twelve hours later, the iNOS mRNA transcription levels of each lectin-treated and untreated cells were measured by reverse transcription–polymerase chain reaction method as described in the text.

iNOS mRNA in PHA-treated RAW264.7 cells, while no significant levels of iNOS mRNA were detected in modeccin-treated and untreated control cells (Fig. 4).

Effects of various monosaccharides on modeccin- and PHA-induced TNF- α secretion in RAW264.7 cells

As shown in Fig. 5, the secretion of TNF- α induced by modeccin was inhibited by galactose and *N*-acetylgalactosamine (GalNAc) in a concentration-dependent manner, whereas mannose and glucose had no inhibitory effect, and even enhanced effects of these monosaccharides at 100 mM were observed. On the other hand, no significant inhibitory effects of the monosaccharides tested were observed in PHA-induced TNF- α secretion, and the levels of TNF- α secreted in PHA-treated RAW264.7 cells were increased in the presence of high concentration of monosaccharides (100 mM). None of the monosaccharide tested alone induced TNF- α secretion (data not shown).

Effect of NH₄Cl on modeccin- and PHA-induced TNF- α secretion in RAW264.7 cells

NH₄Cl is a lysomotropic agent and is known to increase pH in intracellular acidic compartments such as lysosomes

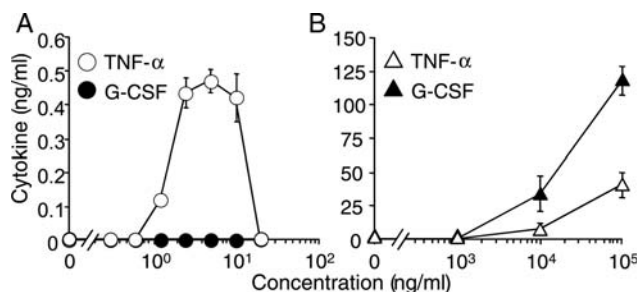


Figure 2 Tumor necrosis factor- α (TNF- α) and granulocyte colony stimulating factor (G-CSF) levels in modeccin- and PHA-treated RAW264.7 cells Adherent RAW264.7 cells (3×10^4 cells per well in 96-well plates) were incubated with the indicated concentrations of modeccin (A) or PHA (B) in DMEM containing 10% of FBS at 37°C. Twenty-four hours later, the supernatant was withdrawn from each well, and subjected to the measurement of each cytokine level by sandwich ELISA method as described in the text. Data were presented as mean \pm SD, $n = 3$.

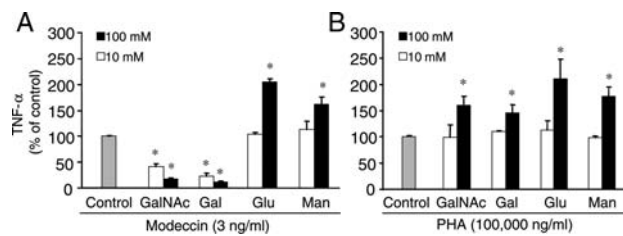


Figure 5 Effects of various monosaccharides on the modeccin- and PHA-induced tumor necrosis factor- α (TNF- α) secretion in RAW264.7 cells. Adherent cells (3×10^4 cells per well in 96-well plates) were pre-incubated with galactose (Gal), *N*-acetylgalactosamine (GalNAc), glucose (Glu) or mannose (Man) (final concentration 10 or 100 mM) for 1 h in DMEM containing 10% of FBS at 37°C, and then modeccin (final concentration 3 ng/ml) (A) or PHA (final concentration 100,000 ng/ml) (B) was added. Twenty-four hours later, the supernatant was withdrawn from each well, and subjected to the measurement of TNF- α levels by sandwich ELISA method as described in the text. Data were presented as mean \pm SD, $n = 3$. * $P < 0.05$ between with and without monosaccharide.

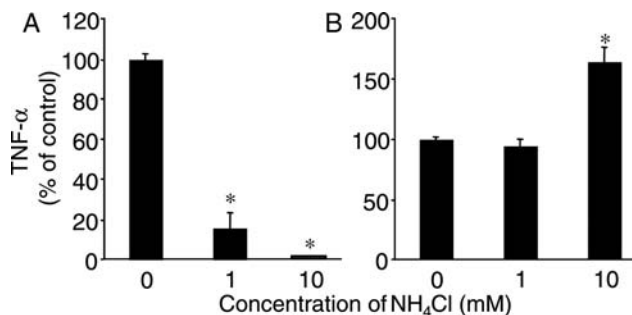


Figure 6 Effects of NH_4Cl on the modeccin- and PHA-induced tumor necrosis factor- α (TNF- α) secretion in RAW264.7 cells. Adherent cells (3×10^4 cells per well in 96-well plates) were pre-incubated with the indicated concentrations of NH_4Cl for 1 h in DMEM containing 10% of FBS at 37°C, and then modeccin (final concentration 3 ng/ml) (A) or PHA (final concentration 100,000 ng/ml) (B) was added. Twenty-four hours later, the supernatant was withdrawn from each well, and subjected to the measurement of TNF- α levels by sandwich ELISA method as described in the text. Data were presented as mean \pm SD, $n = 3$. * $P < 0.05$ between with and without NH_4Cl .

and endosomes in which the pH is maintained at nearly 5.5 under normal culture condition. It has been considered that the transfer of modeccin molecule into acidic compartment during the vesicle trafficking is essential for the intoxication process, and the increase of pH in acidic intracellular compartment by NH_4Cl results in modeccin cytotoxicity inhibition [5]. In agreement with these findings, in the presence of 10 mM NH_4Cl , the toxic effect of modeccin on RAW264.7 cells was almost completely disappeared (data not shown). Furthermore, 10 mM NH_4Cl strongly inhibited modeccin-induced TNF- α secretion. On the other hand, no inhibitory effect of NH_4Cl on PHA-induced TNF- α secretion was observed, and in the presence of 10 mM NH_4Cl , PHA-induced TNF- α level was even increased (Fig. 6).

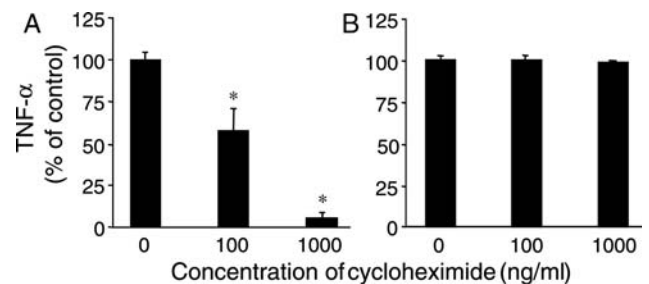


Figure 7 Effects of cycloheximide (CHX) on the modeccin- and PHA-induced tumor necrosis factor- α (TNF- α) secretion in RAW264.7 cells. Adherent cells (3×10^4 cells per well in 96-well plates) were pre-incubated with the indicated concentrations of CHX for 1 h in DMEM containing 10% of FBS at 37°C, and then modeccin (final concentration 3 ng/ml) (A) or PHA (final concentration 100,000 ng/ml) (B) was added. Twenty-four hours later, the supernatant was withdrawn from each well, and subjected to the measurement of TNF- α levels by sandwich ELISA method as described in the text. Data were presented as mean \pm SD, $n = 3$. * $P < 0.05$ between with and without CHX.

Effect of cycloheximide (CHX) on modeccin- and PHA-induced TNF- α secretion in RAW264.7 cells

It has been reported that type 2 RIPs can initiate the ribotoxic stress response after the specific attack on the ribosomal target molecules; furthermore, it has been shown that intact ribosomes with active translational activity are required to initiate ribotoxic stress responses [24,25]. To see whether the ribotoxic stress response was involved in the induction of TNF- α secretion by modeccin, the effect of CHX on the TNF- α level induced by modeccin was examined. As shown in Fig. 7, CHX inhibited modeccin-induced TNF- α secretion in a concentration-dependent manner, while CHX showed no inhibitory effect on PHA activity.

Effect of various kinase inhibitors on modeccin- and PHA-induced TNF- α secretion in RAW264.7 cells

To ascertain whether MAP kinase pathways were involved in TNF- α secretion in RAW264.7 cells treated with modeccin or PHA, the effects of PD98059, SB202190 and SP600125, which are specific inhibitors for ERK, p38, and JNK MAP kinase, were examined. As shown in Fig. 8, p38 MAP kinase inhibitor strongly inhibited TNF- α secretion in modeccin-treated RAW264.7 cells, while other inhibitors had almost no effects. PHA-induced TNF- α secretion was also partly inhibited by p38 MAP kinase inhibitor, while in the case of PHA, ERK inhibitor showed the most potent effect. Protein tyrosine kinase inhibitor, piceatannol, showed a slight but significant inhibitory effect on PHA-induced TNF- α secretion, but had no effect on modeccin-induced TNF- α secretion. The cytotoxicity assay for these inhibitors indicated that at least the concentrations used in this experiment were far less toxic to RAW264.7 cells (Fig. 9).

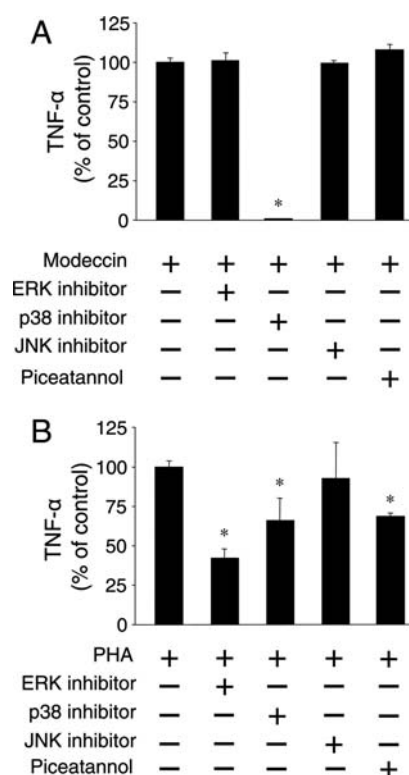


Figure 8 Effects of various kinase inhibitors on the modeccin- and PHA-induced tumor necrosis factor- α (TNF- α) secretion in RAW264.7 cells. Adherent cells (3×10^4 cells per well in 96-well plates) were pre-incubated with three MAP kinase inhibitors (final concentration 15 μ M) for ERK (PD98059), p38 (SB202190), JNK (SP600125) or with piceatannol (final concentration 100 μ M) for 1 h in DMEM containing 10% of FBS at 37°C, and then modeccin (final concentration 3 ng/ml) (A) or PHA (final concentration 100,000 ng/ml) (B) was added. Twenty-four hours later, the supernatant was withdrawn from each well, and subjected to the measurement of TNF- α levels by sandwich ELISA method as described in the text. Data were present as mean \pm SD, $n = 3$. * $P < 0.05$ between with and without inhibitors.

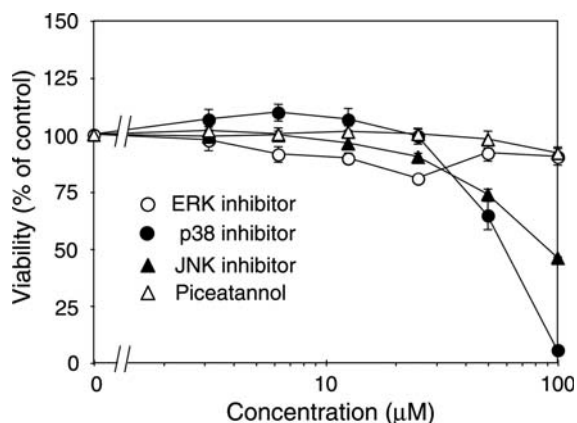


Figure 9 Cytotoxic effects of various kinase inhibitors on RAW264.7 cells. RAW264.7 cells (3×10^4 cells per well in 96-well plates) were incubated with the indicated concentrations of ERK (PD98059), p38 (SB202190), JNK (SP600125), MAP kinase inhibitor or piceatannol in DMEM containing 10% of FBS at 37°C. Twenty-four hours later, the viabilities of the cells were examined by MTT assay as described in the text. Data were present as mean \pm SD, $n = 3$.

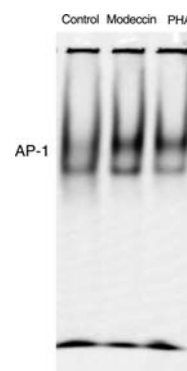


Figure 10 Effects of modeccin and PHA on activator protein 1 (AP-1)-DNA binding in RAW264.7 cells. RAW264.7 cells (2×10^6 cells per well in 6-well plates) were treated with modeccin (final concentration 3 ng/ml) or PHA (final concentration 100,000 ng/ml) in DMEM containing 10% of FBS at 37°C for 5 h. The untreated cells were used as a control. Five hours later, nuclear extracts were examined in EMSA for binding to an oligonucleotide probe corresponding to a consensus AP-1-binding sequence as described in the text.

Effects of modeccin and PHA on activator protein 1 (AP-1)-DNA binding in RAW264.7 cells

AP-1 is an important transcription factor that modulates gene expression of various inflammatory cytokines. To determine whether the PHA- and modeccin-induced TNF- α occurred through transcription factor signaling pathways, EMSA was performed. Nuclear extracts from RAW264.7 cells treated with PHA or modeccin were subjected to EMSA using oligonucleotide probe corresponding to the consensus binding site for AP-1. As shown in Fig. 10, both PHA and modeccin elicited the increase of AP-1-DNA binding activities.

Discussion

Early studies on cytotoxic lectins including type 2 RIPs were mainly focused on the cytotoxic mechanisms. Recent studies, however, have demonstrated that some cytotoxic lectins induce the release of TNF- α and interleukin-1 β in human peripheral-blood mononuclear cells [26,27]. In addition, it has been reported that some bacterial toxins, such as verotoxin, which has the same enzymatic activity and intracellular vesicle trafficking pathway as type 2 RIPs, induce cytokines secretion, and it is considered that the toxin-mediated release of cytokines may have a role in the pathogenesis of the toxin-producing bacteria [28,29]. Mistletoe lectin belonging to type 2 RIP has also been reported to induce TNF- α secretion from human monocytes and T cells [30,31]. Thus, it seems likely that cytotoxic lectins especially type 2 RIPs have an ability to induce multiple cytokines from various cell types as a common biochemical feature.

Modeccin is also a toxic lectin belonging to type 2 RIP. Although modeccin has quite similar structural and functional features to abrin and ricin, previous studies have suggested that there are some modeccin-specific intoxication processes [5]. In contrast to extensive studies on abrin and ricin, the available information regarding biochemical activities of modeccin is limited. Based on these circumstances, in this study, we conducted a comparative study between modeccin and PHA in terms of the induction of cytokines secretion and NO production in RAW264.7 cells. Since PHA has similar sugar specificity to modeccin, we thought it is of interest to conduct comparative studies between modeccin as a representative type 2 RIP and PHA as a toxic lectin without ribosome-inactivation subunit.

Modeccin induced TNF- α secretion from RAW264.7 cells in a dose-dependent manner, but exhibited a bell-shape profile [Fig. 2(A)]. PHA had showed no significant cytotoxicity on RAW264.7 cells up to 100,000 ng/ml (Fig. 1) and induced much higher level of TNF- α than that of modeccin; the higher level of TNF- α was induced along with the increase of the concentration (Fig. 2).

In the presence of galactose or GalNAc, TNF- α secretion induced by modeccin was inhibited in a concentration-dependent manner (Fig. 5). On the other hand, the levels of TNF- α secreted in modeccin-treated cells were rather increased in the presence of high concentration (100 mM) of glucose and mannose. Unexpectedly, all the monosaccharides tested showed enhancement effects on PHA-induced TNF- α secretion at high concentration (100 mM) rather than inhibition. Although the exact reason for the inability of galactose or GalNAc to inhibit PHA-induced TNF- α secretion is unknown now, this may be due to the extremely high concentration of PHA (100,000 ng/ml), and that actual cell surface carbohydrate moiety linked with induction of TNF- α secretion may be recognized by PHA with higher affinity than these monosaccharides. Probably, 10 mM specific monosaccharides may not be enough to cause inhibitory effect, while side effects of monosaccharide may precede the inhibitory effect on lectin binding at 100 mM. In general, lectins exert their biological activities through the recognition of the specific carbohydrate moieties on the target cell surface. However, there seems to be some exceptions. For instance, it has been reported that heat-denatured *Abrus* agglutinins, which lost any sugar binding activity, acted as an adjuvant to induce antigen-specific humoral immune response in rat [32] and also stimulated murine peritoneal macrophages to release NO, superoxide anion, IL-1 and TNF [33]. Thus, in the case of PHA, there is also a possibility that such mechanism might be involved.

Regarding the enhancement effect of monosaccharides especially glucose and mannose on TNF- α secretion, it has

been reported that glucose metabolism is linked to the regulation of inflammatory mediator gene expression [34]. Therefore, high concentration of monosaccharides may somewhat influence the TNF- α secretion itself rather than acting as a competitor for lectin binding on cell surface. Further studies are needed to clarify these points.

Interestingly, in addition to TNF- α , PHA simultaneously induced G-CSF secretion in RAW264.7 cells even with much higher level than that induced by TNF- α (Fig. 2): to our knowledge, this is the first report indicating that PHA can induce G-CSF in RAW264.7 cells. In contrast, modeccin did not induce G-CSF. Furthermore, PHA induced the production of NO in RAW264.7 cells, while no significant level of NO was detected in the modeccin-treated cells (Fig. 3). These results suggested that there were some differences in the ways of modeccin and PHA to stimulate RAW264.7 cells leading to the secretion of bioactive mediators. The intracellular signaling pathways provoked by modeccin may not be connected with G-CSF secretion and NO production, or even if connected, the signals may not be strong enough to induce detectable levels of G-CSF and NO.

To further analyze the underlying mechanisms of TNF- α secretion in modeccin- and PHA-treated RAW264.7 cells, we examined the effects of various inhibitors. NH₄Cl potentially inhibited modeccin-induced TNF- α secretion. However, no significant inhibitory effect of NH₄Cl on PHA activity was observed, and in the presence of 10 mM NH₄Cl, even slightly enhanced TNF- α secretion was observed in PHA-treated cells (Fig. 6). It has been reported that NH₄Cl and monensin, which increase the pH of intracellular acidic vesicular compartments, block transfer of the modeccin A chain from intracellular vesicles to the cytosol, implying that modeccin requires a low pH within vesicles for the enzymatic A subunit to penetrate a vesicle membrane [5]. In fact, the cytotoxic effect of modeccin on RAW264.7 cells was almost completely inhibited by NH₄Cl (data not shown). These results suggest that the ribosomal attack by modeccin through the A-chain is essential for the modeccin-induced TNF- α secretion. The mechanism of the enhancing effect of NH₄Cl on PHA-induced TNF- α secretion is unclear now, but NH₄Cl-induced increase in lysosomal pH may influence the TNF- α secretion process itself. NH₄Cl and other weak base amines have been reported to modulate the secretion process of TNF- α in lipopolysaccharide-stimulated macrophages [35,36]. Thus, opposite effects of NH₄Cl on modeccin and PHA may reflect the different mechanisms of these lectins to induce TNF- α secretion.

CHX, a protein synthesis inhibitor, also inhibited modeccin-induced TNF- α secretion, while no significant effect of CHX on PHA activity was observed (Fig. 7). It has been proposed that intact ribosome with active

translational function is essential for the initiation of so-called ribotoxic stress response, and CHX can prevent the initiation of ribotoxic stress-mediated signaling [24,25]. Thus, it seems likely that the activity of modeccin to induce the secretion of TNF- α in RAW264.7 cells is mainly attributable to its ability to trigger a ribotoxic stress response via the specific attack of the A-chain on ribosomes.

Regarding MAP kinase pathways, our results obtained using three specific MAP kinase inhibitors suggested that p38 MAP kinase was deeply involved in modeccin-induced TNF- α secretion in RAW264.7 cells. Although PHA-induced TNF- α secretion was also partly inhibited by p38 MAP kinase inhibitor, ERK inhibitor showed more potent inhibitory effect on PHA activity (Fig. 8). These results suggest that MAP kinase systems involved in the intracellular-signaling pathway leading to TNF- α secretion are different between modeccin and PHA. Different extent of inhibitory effects of piceatannol, a protein tyrosine kinase inhibitor, on PHA- and modeccin-induced TNF- α secretion may also reflect the different kinase pathways involved in the cytokine secretion by these lectins, although the exact target molecules of this inhibitor is still unclear (Fig. 8).

In addition to MAP kinases, AP-1 has been shown to be responsible for the endotoxin-induced production of a wide array of inflammatory molecules, including IL-1 β , IL-6, TNF- α and iNOS [37]. Consistent with these findings, significant increase in the AP-1–DNA-binding activities in PHA- and modeccin-treated RAW264.7 cells were observed (Fig. 10). Although the exact linkage between MAP kinase systems and AP-1 is still unclear, AP-1 may play a crucial role in the pathway leading to TNF- α secretion induced by PHA and modeccin. Further studies are required to clarify the relationship between MAP kinase systems and AP-1 activation in RAW264.7 cells treated with these lectins.

In conclusion, our results demonstrated that there are significant differences between modeccin and PHA in terms of macrophage activation, (i) both lectins induced TNF- α secretion in RAW264.7 cells, but the dose–response profiles and the levels of TNF- α induced were quite different, (ii) PHA induced the secretion of G-CSF and the production of NO simultaneously, but modeccin did not and (iii) the induction of TNF- α secretion by modeccin and PHA were influenced differently by NH₄Cl, CHX and three MAP kinase inhibitors. It is conceivable that these differences mainly stem from the different ways of modeccin and PHA to stimulate macrophages. Further studies are required to clarify the exact action mechanism of modeccin and PHA, including the intracellular signaling pathways involved in the activation of macrophages.

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