Comparative Study on Modeccin- and Phytohemagglutinin (PHA)-Induced Secretion of Cytokines and Nitric Oxide (NO) in RAW264.7 Cells

Daekyung Kim[1], Yasuhiro Yamasaki[2], Zedong Jiang[2], Yuki Nakayama[2], Tomohiro Yamanishi[2], Kenichi Yamaguchi[2], and Tatsuya Oda[2]*

[1]Jeju Center, Korea Basic Science Institute (KBSI), 66 Jejudaehakno, Jeju-Si, Jeju Special Self-Governing Province 690-756, Korea

[2]Division of Biochemistry, Faculty of Fisheries, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521

*Corresponding address. Tel: +81-95-819-2831; Fax: +81-95-819-2799; E-mail: t-oda@nagasaki-u.ac.jp

Running title Lectin-induced activation of macrophages

We studied the effects of cytotoxic lectins, modeccin and phytohemagglutinin (PHA), on mouse macrophage cell line RAW264.7 in terms of the induction of inflammatory mediators. 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. NH_4Cl (a lysomotoropic 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 NH_4Cl. In addition, the inhibition profiles of modeccin-induced TNF-α secretion by various kinase inhibitors were different from those of PHA. These results suggest 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

Introduction

Lectins are proteins or glycoproteins that are capable of specific recognition and binding to carbohydrate moieties of glycoproteins or glycolipids. Thus, lectins are generally useful tools in studies of cell surface carbohydrate structures and in structural studies of oligosaccharides of glycoconjugates [1]. Many lectins possess various biological activities *in vitro* and *in vivo*, and 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. For instance, it has been reported that the binding of some lectins on the cells involving in the innate immune system can result in cytokine secretion [2, 3].

Modeccin is a toxic lectin present in the roots of the South-African plant, *Modecca digitata*. This toxic lectin contains structurally and functionally different two 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*-glycosidase 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 NH_4Cl, which elevates the intravesicular pH, inhibits the cytotoxicity of modeccin, but enhances the cytotoxicity of ricin, suggesting that modeccin requires NH_4Cl senseitive 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 TNF-α 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), TNF-α, 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 in terms of induction of secretion of inflammatory mediators 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 leading to TNF-α secretion is distinct from that of PHA that can induce G-CSF secretion and NO production in addition to TNF-α secretion. Different responses to various inhibitors were also observed between modeccin- and PHA-induced TNF-α secretions in RAW264.7 cells.

Materials and Methods

Materials

Phytohemagglutinin (PHA) was purchased from Sigma Chemical Co. (St. Luis, MO, USA). Modeccin was obtained from Inland Laboratories (Austin, TX, USA). *N*-nitro-L-arginine methyl ester (L-NAME) was purchased from Dojindo Laboratories (Kumamoto, Japan). PD98059, SB202190, and SP600125, which are specific inhibitor for extracellular regulated kinase (ERK), p38 mitogen-activated protein (MAP) kinase, and c-jun NH_2-terminal kinase (JNK), respectively, were obtained from Calbiochem (La Jolla, CA, USA). Piceatannol (protein tyrosine kinase inhibitor) was purchased from Sigma Chemical Co. (St. Luis, MO, USA). The activator protein 1 (AP-1) consensus IRDye® 700 Infrared dye labeled oligonucleotide probe was purchased from LI-COR Bioscience (Lincoln, NE 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 (Rockville, MD, USA), and cultured in CO_2 (5%)-incubator 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 μg/ml).

Measurement of cytotoxicity

The cytotoxic effects of lectins on RAW264.7 cells were measured by the MTT assay. In brief, adherent RAW264.7 cells (3×10^4 cells per well in 96-well plates) were cultured with varying concentrations of modeccin (final 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 multiwell 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 TNF-α 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 two-fold dilution of murine recombinant cytokines. When the effects of various monosaccharides (final 10 or 100 mM), NH_4Cl (final 1 or 10 mM), cycloheximide (final 100 or 1,000 ng/ml), three different MAP kinase inhibitors (final 15 μM), or piceatannol (final 100 μM) on the TNF-α levels induced by the lectins were examined, the adherent cells (3×10^4 cells per well in 96-well plates) were preincubated with each reagent for 1 h at 37℃ in the growth medium, and then modeccin (final 3 ng/ml) or PHA (final 100,000 ng/ml) was added. After 24 h, the supernatant was withdrawn from each well, and subjected to the measurement of TNF-α level by sandwich ELISA method as described above.

Nitrite assay for the estimation of nitric oxide (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]. Griess reagent (100 μl: 3 mM sulfanilic acid and 30 μM N-1-naphthyl-ethylenediamine dihydrochloride, and 25% glacial acetic acid) was added to 50 μl of each cultured supernatant. After 20 min incubation at room temperature, the optical density was measured at 540 nm using a multiwell scanning spectrophotometer. Calibration curve was made with known concentration of $NaNO_2$ standard solution.

Reverse transcription-polymerase chain reaction

RAW264.7 cells (5 x 10^5 cells per well in 24-well plates) were treated with modeccin (final 3 ng/ml) or PHA (final 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 µg) was reverse transcribed with an oligo dT primer in a 10 µl reaction volumn using PrimeScript® 1st strand cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instruction. PCR was performed with 1 cycle of 70 s at 95℃, 25 cycles of 55 s at 93℃, 45 s at 61℃, 40 s at 72℃ and 1 cycle of 100 s at 72℃, in a 25 µl reaction mixture containing 12.5 µl of GoTag Green Master Mix (promega, Madison, WI, USA), 0.5 µl of forwad and reverse iNOS primers (1µM each) or β-actin primers (10 pM each), 0.5 µl of 1st strand cDNA and 11 µl nuclease-free water. The primer sequences were 5' -CAACCAGTATTATGGCTCCT-3' (forward) and 5' -GTGACAGCCCGGTCTTTCCA-3' (reverse) for mouse iNOS and 5' -GGAGAAGATCTGGCACCACACC-3' (forward) and 5' -CCTGCTTGCTGATCCACATCTGCTGG-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 µg/ml 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 x 10^6 cells per well in 6-well plates) were treated with modeccin (final 3 ng/ml) or PHA (final 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, Lincoln, NE USA) according to manufacturer's instructions. Briefly, 5 µg of nuclear extract was incubated with 1 µl of IRD® 700 Infrared dye labeled double-stranded oligonucleotides, 2 µl of 10 x binding buffer, 2.5 mM DTT, 0.25% Tween-20, and 1µg of poly

(dl-dC) in a total volume of 20 μl for 30 min at room temperature in dark. Samples were separated on a 4% polyacrylamide gel in 0.25 x 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, Lincoln, NE USA). AP-1 IRDye® 700 infrared dye labeled oligonucleotide's sequences of the double-stranded DNA probes used were as follows: 5′ -CGCTTGA<u>TGACTCA</u>GCCGGAA-3′ and 3′ -GCGAACT<u>ACTGAGT</u>CGGCCTT-5′ .

Statistical analysis

All the experiments were repeated at least three times. The results were expressed as a mean ± 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, IL, USA). A p-value < 0.05 was considered statistically significant.

Results

Cytotoxic effects of modeccin and PHA on RAW264.7 cells

Fig. 1 shows typical dose-dependent curves of modeccin and PHA. Modeccin showed highly cytotoxic effect on RAW264.7 cells. Based on the results, the IC_{50} value of modeccin was estimated to be 2.0-4.0 ng/ml depending on the experiments. PHA showed no significant cytotoxicity up to 100,000 ng/ml on this cell line.

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 as compared to modeccin, and increased level of TNF-α along with the increase in the 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)**].

Nitric oxide (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 NO synthase (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 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 NH_4Cl on modeccin- and PHA-induced TNF-α secretion in RAW264.7 cells

NH_4Cl is a lysomotropic agent, and is known to increase pH in intracellular acidic compartments such as lysosomes and endosomes in which the pH is maintained at nearly 5.5 under normal culture condition. It has been considered that transfer of modeccin molecule into acidic compartment during the vesicle trafficking is essential for the intoxication process, and the increase in pH in acidic intracellular compartment by NH_4Cl results in the inhibition of modeccin cytotoxicity [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)**.

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 is 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 are involved in the secretion of TNF-α in RAW264.7 cells treated with modeccin or PHA, the effects of PD98059, SB202190, and SP600125, which are specific inhibitor for ERK, p38, and JNK MAP kinase, respectively, were examined. As shown in **Fig. 8**, p38 MAP kinase inhibitor strongly inhibited the secretion of TNF-α 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, but 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**).

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, electrophoretic mobility shift assay (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 increases in 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-1β 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 the secretion of cytokines, 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 the secretion of TNF-α 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, 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 that had showed no significant cytotoxicity on RAW264.7 cells up to 100,000 ng/ml **(Fig. 1)**, induced much higher level of TNF-α than that of modeccin, and the higher level of TNF-α was induced along with the increase in the concentration **(Fig. 2)**.

In the presence of galactose or GalNAc, secretion of TNF-α 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 effect 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* agglutinin, 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 lection binding on cell surface. Further studies are needed to clarify these points.

Interestingly, in addition to TNF-α, PHA simultaneously induced the secretion of G-CSF in RAW264.7 cells even with much higher level than that of 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 nitric oxide (NO) in RAW264.7 cells, while no significant level of NO was detected in the modeccin-treated cells **(Fig. 3)**. These results suggest that there are 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. NH_4Cl potently inhibited modeccin-induced TNF-α secretion. However, no significant inhibitory effect of NH_4Cl on PHA activity was observed, and in the presence of 10 mM NH_4Cl, even slightly enhanced TNF-α secretion was observed in PHA-treated cells **(Fig. 6)**. It has been reported that NH_4Cl 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 NH_4Cl (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 NH_4Cl on PHA-induced TNF-α secretion is unclear now, but NH_4Cl-induced increase in lysosomal pH may influence the TNF-α secretion process itself. NH_4Cl and other weak base amines have been reported to modulate the secretion process of TNF-α in LPS-stimulated macrophages [35, 36]. Thus, opposite effects of NH_4Cl 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-1β, 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, (iii) the induction of TNF-α secretion by modeccin and PHA were influenced differently by NH_4Cl, 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.

References

1. Sharon N, Lis H. Lectins as cell recognition molecules. Science 1989, 246: 227-234.

2. Campbell D, Mann BJ, Chadee K. A subunit vaccine candidate region of the *Entamoeba histolytica* galactose-adherence lectin promotes interleukin-12 gene transcription and protein production in human macrophages. Eur J Immunol 2000, 30: 423-430.

3. Fujita Y, Homman KJ, Natori S. Activation of murine macrophage-like cells by granulocytin. Biochem Biophys Res Comm 2000, 275: 850-853.

4. Endo Y, Mitsui K, Motizuki K, Tsurugi K. The mechanism of action of ricin and related toxic lectins on eukaryotic ribosomes. The site and the characteristics of the modification in 28S ribosomal RNA caused by the toxins. J Biol Chem 1987, 262: 5908-5912.

5. Sandvig K, Sundan A, Olsnes S. Evidence that diphtheria toxin and modeccin enter the cytosol from different vesicular compartments. J Cell Biol 1984, 98: 963-970.

6. Sandvig K, van Deurs B. Toxin-induced cell lysis: Protection by 3-methyladenine and cycloheximide. Exp Cell Res 1992, 200: 253-262.

7. Oda T, Komatsu N, Muramatsu T. Cell lysis induced by ricin D and ricin E in various cell lines. Biosci Biotechnol Biochem 1997, 61: 291-297.

8. Oda T, Komatsu N, Muramatsu T. Diisopropylfluorophosphate (DFP) inhibits ricin-induced apoptosis of MDCK cells. Biosci Biotechnol Biochem 1998, 62: 325-333.

9. Griffiths GD, Leek MD, Gee DJ. The toxic plant proteins ricin and abrin induce apoptotic change in mammalian lymphoid tissues and intestine. J Pathol 1987, 151: 221-229.

10. Higuchi S, Tamura T, Oda T. Cross-talk between the pathway leading to the induction of apoptosis and the secretion of tumor necrosis factor-α in ricin-treated RAW264.7 cells. J Biochem 2003, 134: 927-933.

11. Yamasaki C, Nishitaka K, Zeng X-T, Katayama Y, Natori Y, Komatsu N, Oda T, Natori Y. Induction of cytokines by toxins that have an identical RNA *N*-glycosidase activity: Shiga

toxin, ricin, and modeccin. Biochim Biophys Acta-Gen Subj 2004, 1671: 44-50.

12. Kim M, Rao MV, Tweardy DJ, Prakash M, Galili U, Gorelik E. Lectin-induced apoptosis of tumour cells. Glycobiology 1993, 3: 447-453.

13. Abraham RT, Ho SN, Barna TJ, McKean DJ. Transmembrane signaling during interleukin 1-dependent T cell activation. Interaction of signal 1- and singal 2-type mediators with the phosphoinositide-dependent signal transduction mechanism. J Biol Chem 1987, 262: 2719-2728.

14. Benbernon N, Esnault S, Shin HCK, Fekkar H, Guenounou M. Differential regulation of IFN-γ, IL-10 and inducible nitric oxide synthase in human T cells by cyclic AMP dependent signal transduction pathway. Immunology 1997, 91: 361-368.

15. Pahlavani MV. T cell signaling: effect of age. Front Biosci 1998, 3: 125-135.

16. Kesherwani V, Sodhi A. Differential activation of macrophages in vitro by lectin Concanavalin A, Phytohemagglutinin and Wheat germ agglutinin: Production and regulation of nitric oxide. Nitric oxide 2007, 16: 294-305.

17. Wong JH, Ng TB. Isolation and characterization of a glucose/mannose-specific lectin with stimulatory effect on nitric oxide production by macrophages from the emeperor banana. Inter J Biochem Cell Biol 2006, 38: 234-243.

18. Korcheva V, Wong J, Corless C, Iordanov M, Magun B. Administration of ricin induces a severe inflammatory response via nonredundant stimulation of ERK, JNK, and P38 MAPK and provides a mouse model of hemolytic uremic syndrome. Am J Pathol 2005, 166: 323-339.

19. Iwamoto M, Kurachi M, Nakashima T, Kim D, Yamaguchi K, Oda T, Iwamoto Y, Muramatsu T. Structure-activity relationship of alginate oligosaccharides in the induction of cytokine production from RAW264.7 cells. FEBS Lett 2005, 579: 4423-4429.

20. Yamamoto Y, Kurachi M, Yamaguchi K, Oda T. Induction of multiple cytokine secretion from RAW264.7 cells by alginate oligosaccharides. Biosci Biotechnol Biochem 2007, 71:

238-241.

21. Yamanishi T, Hatakeyama T, Yamaguchi K, Oda T. CEL-I, an *N*-acetylgalactosamine (GalNAc)-specific C-type lectin, induces nitric oxide production in RAW264.7 mouse macrophage cell line. J Biochem 2009, 146: 209-217.

22. Zingarelli B, Hake PW, Yang Z, O'Coonor M, Denenberg A, Wong HR. Absence of inducible nitric oxide synthase modulates early reperfusion-induced NF-κB and AP-1 activation and enhances myocardial damage. FASEB J 2002, 16: 327-342.

23. Yamanishi T, Yamamoto Y, Hatakeyama T, Yamaguchi K, Oda T. CEL-I, an invertebrate *N*-acetylgalactosamine-specific C-type lectin, induces TNF-α and G-CSF production by mouse macrophage cell line RAW264.7 cells. J Biochem 2007, 142: 587-595.

24. Iordanov MS, Pribnow D, Magun JI, Dinh T-H, Pearson JA, Chen SL-Y, Magun BE. Ribotoxic stress response: Activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the α-sarcin/ricin loop in the 28S rRNA. Mol Cell Biol 1997, 17: 3373-3381.

25. Shifrin VI, Anderson P. Trichothecene mycotoxins trigger a ribotoxic stress response that activates c-jun N-terminal kinase and p38 mitogen-activated protein kinase and induces apoptosis. J Biol Chem 1999, 274: 13985-13992.

26. Licastro F, Morini MC, Bolognesi A, Stirpe F. Ricin induces the production of tumour necrosis factor-α and interleukin-1β by human peripheral-blood mononuclear cells. Biochem J 1993, 294: 517-520.

27. Hassoun E, Wang X. Ricin-induced toxicity in the macrophage J744A.1 cells: The role of TNF-α and the modulation effects of TNF- α polyclonal antibody. J Biochem Mol Toxicol 2000, 14: 95-101.

28. Schumann J, Tiegs G. Pathophysiological mechanisms of TNF during intoxication with natural or man-made toxins. Toxicology 1999, 138: 103-126.

29. Yamasaki C, Natori Y, Zeng XT, Ohmura M, Yamasaki S, Takeda Y, Natori Y. Induction of

cytokines in a human colon epithelial cell line by Shiga toxin (Stx1) and Stx2 but not by non-toxic mutant Stx1 which lacks *N*-glycosidase activity. FEBS Lett 1999, 442: 231-234.

30. Ribéreau-Gayon G, Dumont S, Muller C, Jung M-L, Poindron P, Anton R. Mistletoe lectin I, II and III induce the production of cytokines by cultured human monocytes. Cancer Lett 1996, 109: 33-38.

31. Gong F, Ma YH, MA AL, Yu QW, Zhang JY, Nie H, Chen XH, Shen BH, Li NL, Zhang DQ. A lectin from Chinese mistletoe increases $\gamma\delta$ T cell-mediated cytotoxicity through induction of caspase-dependent apoptosis. Acta Biochim Biophys Sin 2007, 39: 445-452.

32. Suryakala S, Maiti TK, Sujatha N, Sashidhar RB. Identification of a noval protein adjuvant isolated from *Abrus precatorius*. Food Agri Immunol 2000, 12: 87-96.

33. Tripathi T, Maiti TK. Stimulation of murine macrophages by native and heat-denatured lectin from *Abrus precatorius*. Inter Immunopharmacol 2003, 3: 233-244.

34. Won JS, Im YB, Key L, Singh I, Singh AK. The involvement of glucose metabolism in the regulation of inducible nitric oxide synthase gene expression in glial cells: possible role of glucose-6-phosphate dehydrogenase and CCAAT/enhancing binding protein. J Neurosci 2003, 23: 7470-7478.

35. Jeong JY, Jue DM. Chloroquine inhibits processing of tumor necrosis factor in lipopolysaccharide-stimulated RAW 264.7 macrophages. J Immunol 1997, 158: 4901-4907.

36. Jang CH, Choi JH, Byun MS, Jue DM. Chloroquine inhibits production of TNF-α, IL-1β and IL-6 from lipopolysaccharide-stimulated human monocytes/macrophages by different modes. Rheumatology 2006, 45: 703-710.

37. Jiang JX, Zhang Y, Ji SH, Zhu PF, Wang ZG. Kinetics of mitogen-activated protein kinase family in lipopolysaccharide-stimulated mouse kupffer cells and their role in cytokine production. Shock 2002, 18: 336-341.
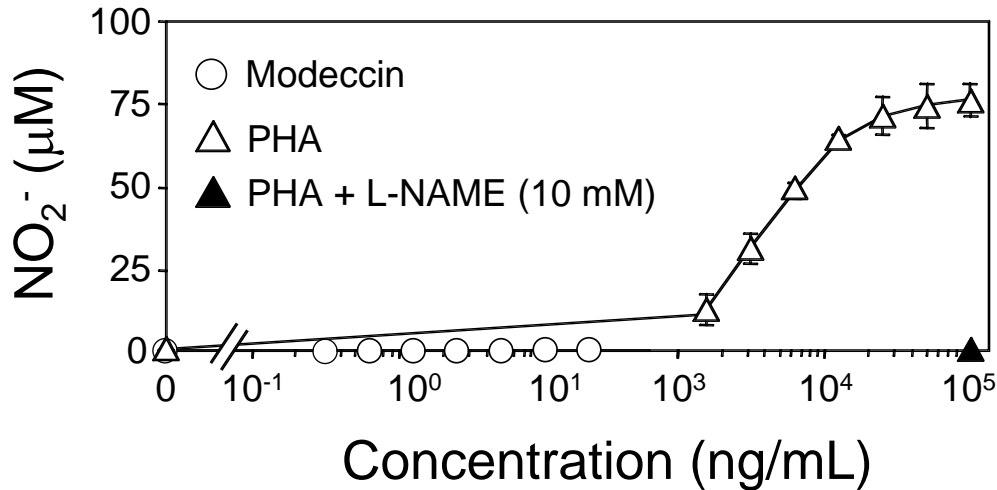
Figure Legends
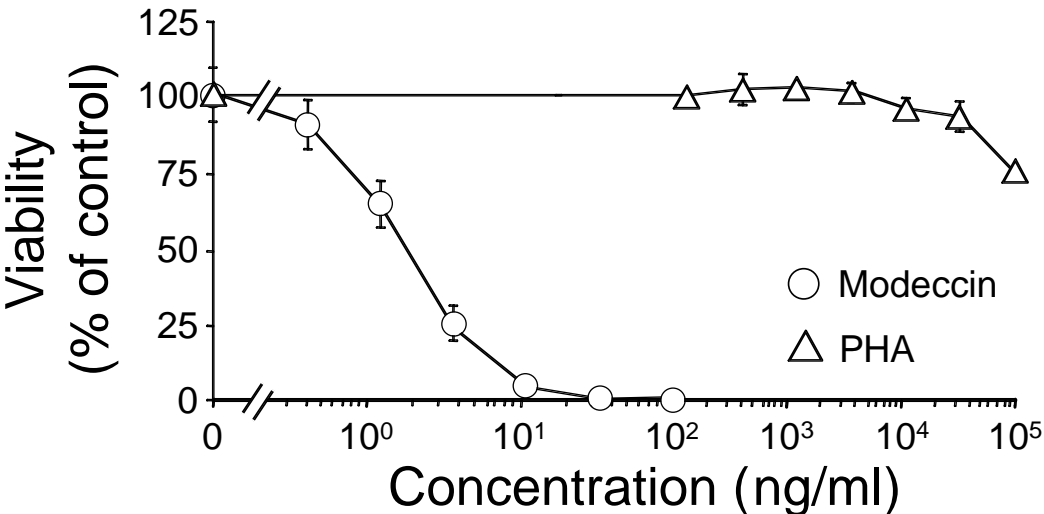
Fig. 1 Cytotoxic effects of modeccin and PHA on RAW264.7 cells

Adherent RAW264.7 cells (3 x 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 represents mean SD, $n = 3$.
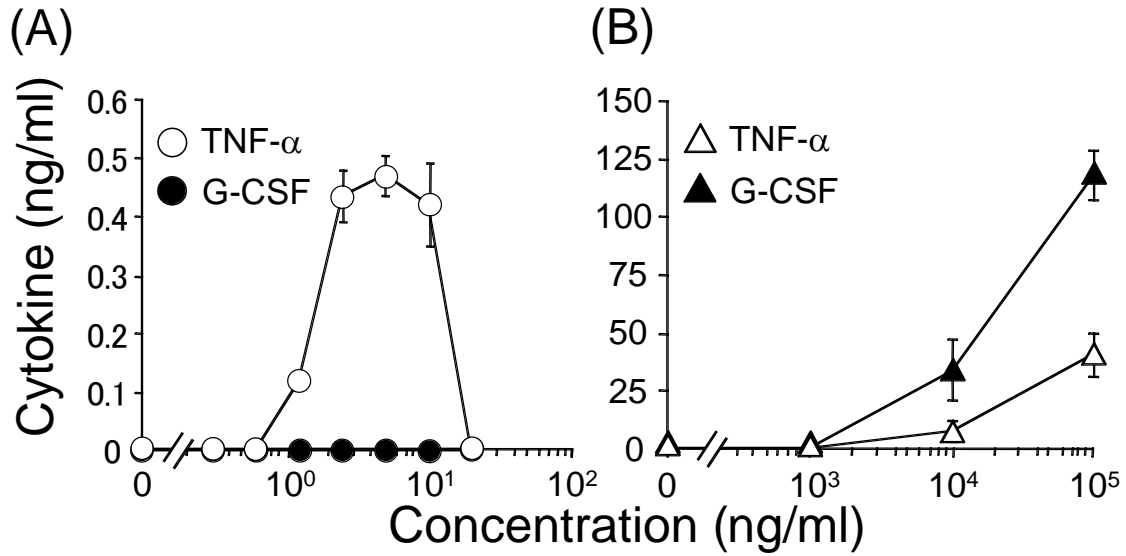
Fig. 2   Tumor necrosis factor-α (TNF-α) and granulocyte colony stimulating factor (G-CSF) levels in modeccin- and PHA-treated RAW264.7 cells

Adherent RAW264.7 cells (3 x 10^4 cells per well in 96-well plates) were incubated with the indicated concentrations of (A) modeccin or (B) PHA in DMEM containing 10% of FBS at 37℃. After 24 h, 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 represents mean SD, *n* = 3.

**Fig. 3 Nitric oxide (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℃. 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 represents mean SD, *n* = 3.

**Fig. 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℃. The untreated cells were used as a control. After 12 h, the iNOS mRNA trascraption levels of each lectin-treated and untreated cells were measured by reverse transcription-polymerase chain reaction method as described in the text.
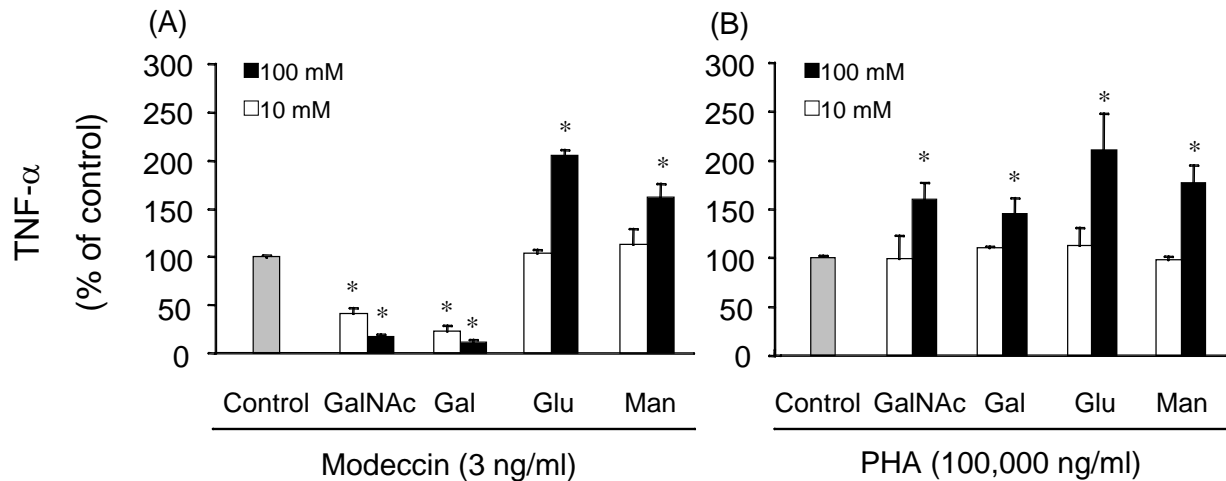
Fig. 5 Effects of various monosaccharides on the modeccin- and PHA-induced tumor necrosis factor-α (TNF-α) secretion in RAW264.7 cells
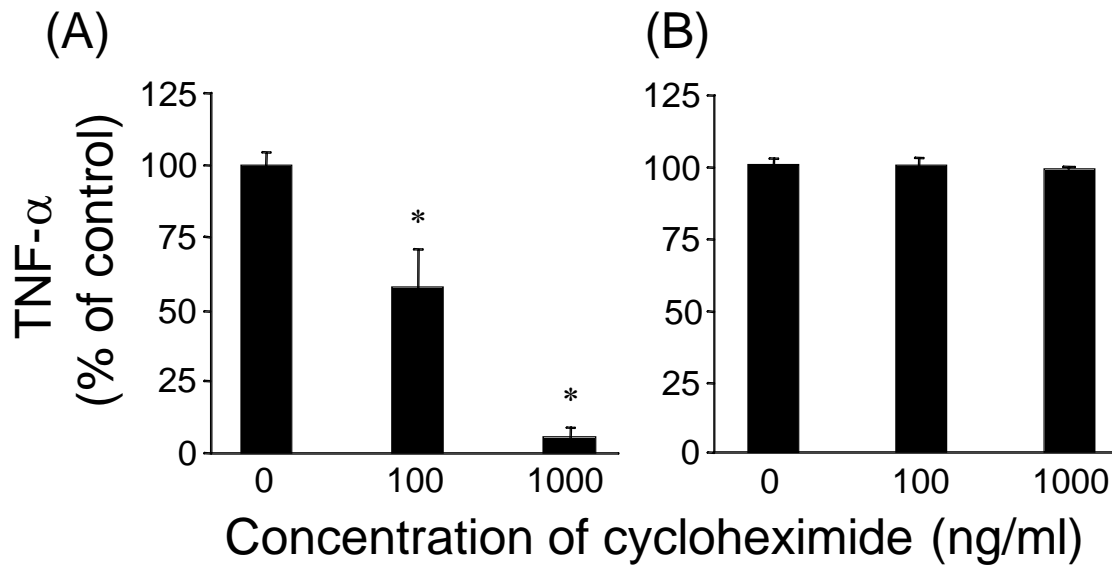
Adherent cells (3×10^4 cells per well in 96-well plates) were preincubated with galactose (Gal), *N*-acetylgalactosamine (GalNAc), glucose (Glu), or mannose (Man) (final 10 or 100 mM) for 1 h in DMEM containing 10% of FBS at 37℃, and then (A) modeccin (final 3 ng/ml) or (B) PHA (final 100,000 ng/ml) was added. After 24 h, 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 represents mean SD, $n = 3$. Asterisks indicate significant differences between with and without monosaccharide ($p < 0.05$).
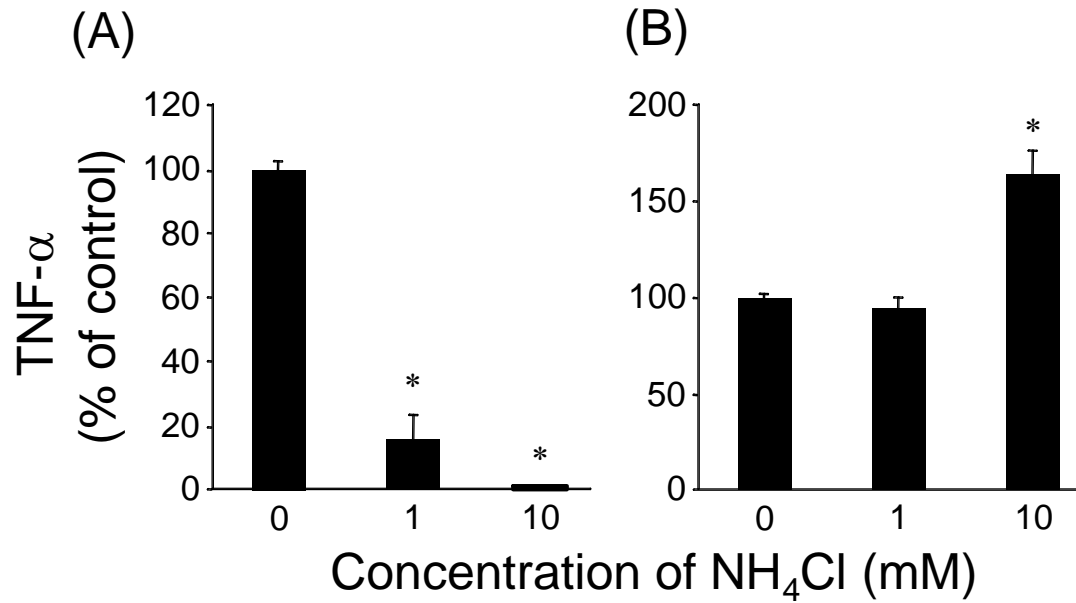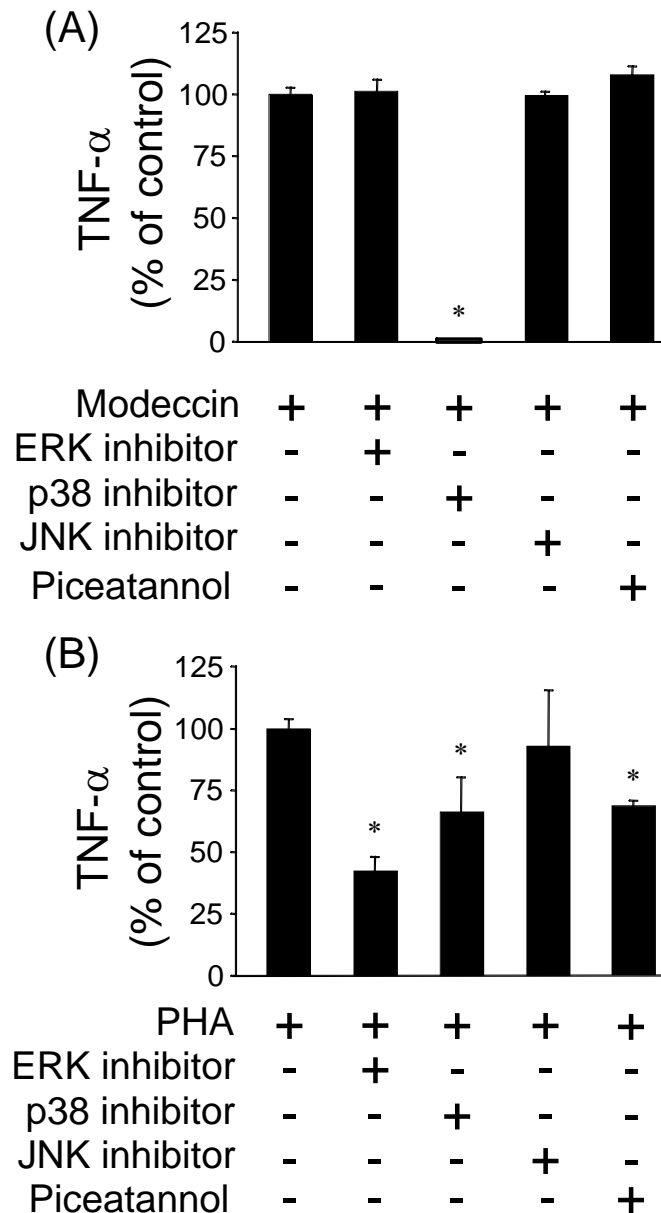
Fig. 6 Effects of NH₄Cl on the modeccin- and PHA-induced tumor necrosis factor-α (TNF-α) secretion in RAW264.7 cells

Adherent cells (3×10^4 cells per well in 96-well plates) were preincubated with the indicated concentrations of NH$_4$Cl for 1 h in DMEM containing 10% of FBS at 37°C, and then (A) modeccin (final 3 ng/ml) or (B) PHA (final 100,000 ng/ml) was added. After 24 h, 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 represents mean SD, $n = 3$. Asterisks indicate significant differences between with and without NH$_4$Cl ($p < 0.05$).

Fig. 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 x 10^4 cells per well in 96-well plates) were preincubated with the indicated concentrations of CHX for 1 h in DMEM containing 10% of FBS at 37℃, and then (A) modeccin (final 3 ng/ml) or (B) PHA (final 100,000 ng/ml) was added. After 24 h, 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 represents mean SD, $n = 3$. Asterisks indicate significant differences between with and without CHX ($p < 0.05$).

(A)

TNF-α (% of control)

Modeccin	+	+	+	+	+
ERK inhibitor	-	+	-	-	-
p38 inhibitor	-	-	+	-	-
JNK inhibitor	-	-	-	+	-
Piceatannol	-	-	-	-	+

(B)

TNF-α (% of control)

PHA	+	+	+	+	+
ERK inhibitor	-	+	-	-	-
p38 inhibitor	-	-	+	-	-
JNK inhibitor	-	-	-	+	-
Piceatannol	-	-	-	-	+

Fig. 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 x 10^4 cells per well in 96-well plates) were preincubated with of ERK (PD98059), p38 (SB202190), JNK (SP600125) MAP kinase inhibitor (final 15 μM), or piceatannol (final 100 μM) for 1 h in DMEM containing 10% of FBS at 37℃, and then (A) modeccin (final 3 ng/ml) or (B) PHA (final 100,000 ng/ml) was added. After 24 h, 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 represents mean SD, $n = 3$. Asterisks indicate significant differences between with and without inhibitors ($p < 0.05$).
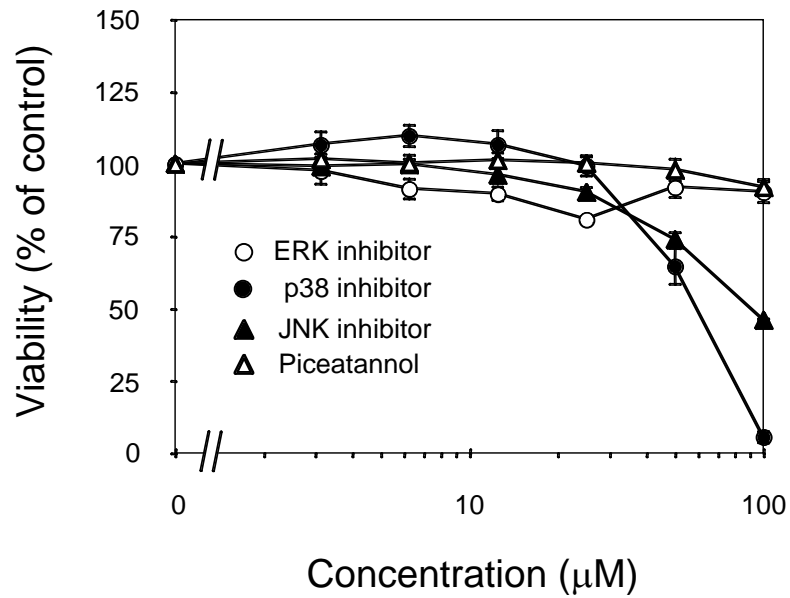
Fig. 9 Cytotoxic effects of various kinase inhibitors on RAW264.7 cells

RAW264.7 cells (3 x 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℃. After 24 h, the viabilities of the cells were examined by MTT assay as described in the text. Data represents mean SD, $n = 3$.

Control Modeccin PHA

AP-1
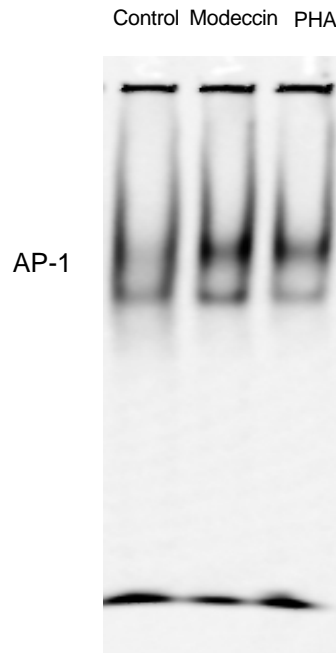
Fig. 10 Effects of modeccin and PHA on activator protein 1 (AP-1)-DNA binding in RAW264.7 cells

RAW264.7 cells (2×10^6 cells per well in 6-well plates) were treated with modeccin (final 3 ng/ml) or PHA (final 100,000 ng/ml) in DMEM containing 10% of FBS at 37℃ for 5 h. The untreated cells were used as a control. After 5 h, nuclear extracts were examined in electrophoretic mobility shift assay (EMSA) for binding to an oligonucleotide probe corresponding to a consensus AP-1-binding sequence as described in the text.