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In Vitro Antitumor and Immunomodulatory Effects of the Protein PCP-3A from Mushroom *Pleurotus citrinopileatus*

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A nonlectin glycoprotein (PCP-3A) newly isolated from the fruit body of edible golden oyster mushroom *Pleurotus citrinopileatus* has been shown to be growth inhibitory against human myeloid leukemic U937 cells in a previous report. There is a well-recognized relation between antitumor activity and immunomodulation. The immunomodulatory activity of PCP-3A was therefore assessed in the present study. Human mononuclear cells (MNC) and the $CD4^+$ T lymphocytes isolated from them were stimulated separately with PCP-3A for various durations and then filtered to obtain the conditioned media (CM). The conditioned medium from MNC (MNC-CM) was proved effective in inhibiting the growth of U937 cells. Increased secretion of cytokines TNF-α, IL-2, and IFN-γ from the stimulated MNC and $CD4^+$ T cells was found in CM. The antibody neutralization test of MNC-CM revealed that the growth inhibition on leukemic U937 cells was related to the elevation in cytokine concentration. We propose that PCP-3A stimulated human MNC to secrete cytokines TNF-α, IL-2, and IFN-γ, which subsequently inhibit the growth of U937 cells, and that PCP-3A may be a possible material for developing into an antileukemia ingredient in health food.

KEYWORDS: Mushroom; cytokine; immunomodulation; glycoprotein; leukemic

INTRODUCTION

Mushrooms have become an important source of bioactive compounds for the treatment of various diseases (*1*−*5*). Some chemical compounds in specific mushrooms exhibit antitumor activity via the modulation of the immune system (*6*). Immunomodulatory activity has been demonstrated for crude extracts and isolated components, including proteins, glycoproteins, polysaccharopeptides, and polysaccharides, from the fruiting bodies, mycelia, spores, and culture media of various mushrooms (*7*).

Glycoproteins are important water-soluble biopolymers for use as a health food ingredient. Food supplements containing fungal glycoproteins are widely sold in health food stores for their effects of immunomodulation, tumor inhibition, etc. (*7*−*11*).

Pleurotus citrinopileatus, commonly called "golden oyster mushroom", belongs to the family Basidiomycete. This mushroom has recently become a popular delicacy in East Asia, including China, Japan, and Taiwan, for its taste and health value. The extract of this mushroom is effective in antioxidation (*12*), cardiovascular disease prevention (*13*), immunomudulation (*14*), the reduction of blood sugar levels (*15*), and antitumor activities (*16*). Recently, a novel nonlectin glycoprotein, PCP-3A, was isolated from the fruiting body and shown to be growth inhibitory against human myeloid leukemic U937 cells in a direct model in vitro (*17*).

There is a well-recognized relation between antitumor activity and immunomodulation. Cytokines are crucial in the induction and regulation of immune responses in cellular level (*18*). Cytokines can be activated by antitumor agents such as glycoprotein. For example, PSK, a heterogeneous mixture of glycoproteins obtained from *Coriolus versicolor* of Basidiomycetes (*9*), was reported to promote the gene expression in human peripheral blood mononuclear cells (MNC) for the secretion of cytokines IL-6, IL-8, TNF-α, etc. in vitro (*19*) and TNF-α and IL-8 in vivo (*20*).

The present study was to investigate the antitumor and immunomodulatory effects of PCP-3A in an indirect model. MNC was incubated with PCP-3A for preparing a conditioned medium (CM). The growth inhibitory activity of CM on U937 cells was evaluated. The cytokine contents in CM were assessed to elucidate the immunomodulatory mechanism and to explain the antitumor effect.

MATERIALS AND METHODS

Isolation of PCP-3A. The procedure referred to our previous work (*17*). The fresh fruiting bodies (200 g) of the mushroom *P. citrinopileatus* were purchased from a local supermarket, cleaned by rinsing several times in tap water, blended with acetone (1:3, w/v) for 1 min using a Waring blender (Cycle blender, Osterizer, Mexico), and then filtered through a sieve to collect the residue. The residue was soaked in 0.5 L of 10% NaCl at 50 °C for 1 h to form a slurry. The slurry was centrifuged at 8500*g* for 30 min to collect the supernatant. Solid ammonium sulfate was added to the supernatant to reach 40% saturation and then centrifuged to discard the precipitate. More ammonium sulfate was added into the supernatant to 80% saturation and then centrifuged again to collect the 40−80% saturation precipitate. The precipitate was dissolved in a small volume of distilled water and then dialyzed against distilled water to obtain the crude mushroom extract.

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The crude extract was applied to a DEAE-Sepharose column (GE Healthcare, Uppsala, Sweden) (2.6 cm × 30 cm), which had previously been equilibrated with 0.05 M Tris-HCl buffer (pH 8.0). The column was then eluted with 0.05 M Tris-HCl buffer (pH 8.0) in two steps. In the first step, the column was eluted with 5 bed volumes of the Tris-HCl buffer for the unadsorbed fractions. In the second step, protein-rich fractions were collected through the elution with 5 bed volumes of 0−0.5 M NaCl gradient Tris-HCl buffer. The growth inhibitory activity of each fraction was evaluated. The fraction with the highest activity was further fractionated by gel filtration in a 1.6 cm × 100 cm Sephacryl S-300 column (GE Healthcare) eluted with 3 bed volumes of the Tris-HCl buffer. The protein-rich subfraction that retained the highest growth inhibitory activity was freeze-dried to recover PCP-3A for the following experiments.

Analysis of Amino Acid Composition and Determination of N-Terminal Amino Acid Sequence. The amino acid analysis of PCP-3A was performed using a Waters Pico-Tag system (Milford, MA). The values of threonine and serine were corrected for hydrolysis loss by extrapolating to zero time relative to alanine. The tryptophan content was separately determined from the absorbance at 280 and 288 nm in 6 M guanidine HCl. The N-terminal sequence of PCP-3A was determined by automated Edman degradation using a model 477A amino acid sequencer from Applied Biosystems (Foster City, CA) with the manufacturer's standard program and chemicals.

Preparation of CM. Human peripheral blood was obtained from three normal adult volunteers (ages between 22 and 25) with informed consent. MNC was recovered from the blood of each person by density centrifugation (400*g*, 30 min) in a Ficoll−Hypaque solution (1.077 g/mL) (*21*). Fetal bovine serum (FBS) (Hyclone, Logan, UT) was heated at 56 °C in a water bath for 30 min to inactivate the heat-labile complement proteins. MNC was suspended in 20 μg/mL polymyxin B (PMB, Sigma)/2 mM glutamine/10% heat-inactivated FBS/RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) in the density of 1.5×10^6 cells/mL. Polymyxin was to prevent the contamination of endotoxin. Each 10 μL aliquot of PCP-3A (5−25 μg/mL of medium) in phosphate-buffered saline (PBS) was mixed with a 2 mL aliquot of the MNC suspension, incubated at 37 °C in a humidified 5% CO_2 incubator for 1, 2, and 3 days, and filtered through a 0.45 μm membrane to obtain the conditioned media (CM) PCP-3A-MNC-CM-1, -2, and -3, respectively. The CM were stored at −80 °C until use. Phytohemeagglutinin (PHA) (5 μg/mL of medium) and PBS were used to prepare CM PHA-MNC-CM (positive group) and PBS-MNC-CM (negative group), separately.

Preparation of $CD4^+$ T Lymphocytes. MNCs were prepared using the same technique as above-described for MNC. Purified $CD4^+$ T cells were negatively selected using IMag Human CD4 T Lymphocyte Enriched Set (BD Biosciences, San Jose, CA). Briefly, PBMC were incubated in a mixture of biotinylated Abs not binding CD4 T lymphocytes. Streptavidin particles were added to the cell suspension. CD4 T cells were then collected following magnetic depletion.

Cell Cultivation and Treatments. U937 cells were cultured in 1% glutamine/10% FBS/RPMI 1640 medium and maintained in the exponential phase of growth. The cells were incubated in 35 mm Petri dishes at an initial concentration of 1×10^5 cells/mL in the presence (sample group) or absence (control group) of 20% (v/v) MNC-CM. In addition to the above-described indirect method of inhibition, the direct method that incubated U937 cells in the presence of PCP-3A in culturing medium was also applied. In both the indirect and the direct methods, adherent cells in 3 day cultures were collected by gently rubbing the dishes with a rubber policeman, and the cell numbers were counted using the Trypan Blue Dye exclusion test (*22*) to determine the growth inhibition (%) (*21*): growth inhibition (%) (1 − cell number in sample group/cell number in control group) × 100%. Three separate experiments were performed. Each was tested in duplicate.

Evaluation of Cytokine Production. The contents of cytokines TNF-α, IFN-γ, IL-2, and IL-4 in CM were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits from BioSource (Carlsbad, CA).

Antibody Neutralization. PCP-3A-MNC-CM-1 was preincubated at 37 °C for 90 min with 30 μg/mL of various cytokine-neutralizing antibodies (Epitomics, Burlingame, CA), including anti- TNF-α, anti-IFN-γ and anti-IL-2 in combination or alone. PCP-3A-MNC-CM-1 preincubated with normal rabbit serum served as the control. The preincubated PCP-3A-MNC-CM-1 samples were added to U937 cell cultures and incubated for 3 more days to examine the cell growth.

Table 1. Amino Acid Composition of PCP-3A[a]

amino acid	content (%)
Asx	11.75
Thr	5.3
Ser	5.05
Glx	9.86
Pro	9.43
Gly	7.33
Ala	7.82
Cys	0.47
Val	6.68
Ile	5.22
Leu	tr.
Tyr	3.72
Phe	6.28
Met	3.43
Lys	3.76
His	2.64
Try	ND
Arg	2.08

[a] Glx, sum of Glu and Gln; Asx, sum of Asp and Asn; ND, not detectable; and tr., trace.

Table 2. Comparison between PCP-3A and Other Reported Agaricales Glycoproteins on N-Terminal Amino Acid Sequencing

species	N-terminal sequence	reference
P. citrinopileatus	AGNPEENAPNW	this study
P. citrinopileatus	QYSQMAQVME	*16*
Pholiota adipose	YSVTTPNSVKGGTNQPGA	*23*
A. bisporus	MGGSGTSGSL	*24*
F. velutipes	TSLTFQLAYL	*25*
A. aegerita	NISAGTSVDL	*26*
C. cinerea	IPLEGTFGDR	*27*
P. cornucopiae	SDSTWTFAML	*28*
M. oreades	YILDGEYLVL	*29*

Statistical Analysis. The results were expressed as means ± standard deviations. Statistical comparisons were made by one-way analysis of variance (ANOVA), followed by Duncan's multiple-comparison test. Differences were considered significant when the p values were < 0.05.

RESULTS AND DISCUSSION

Amino Acid Composition and N-Terminal Amino Acid Sequence. The amino acid composition of PCP-3A is presented in **Table 1**. There are high contents of acidic amino acids, including proline (9.43%), alanine (7.82%), glycine (7.33%), valine (6.68%), and phenylalaine (6.28%); low contents of histidine (2.64%), arginine (2.08%), and cysteine (0.47%); and traces of leucine. The present study identified a sequence of 11 amino acids, AGNPEENAPNW, in the N terminus of PCP-3A. Such an N-terminal amino acid sequence was not found in mushroom proteins listed in GeneBank database (http://www.ncbi.nlm.nih.gov/BLAST/). No resemblance with any of the published N-terminal amino acid sequences of glycoproteins from Agaricales mushrooms, including *Agaricus bisporus*, *Agrocybe aegerita*, *Coprinopsis cinerea*, *Flammulina velutipes*, *Laccaria bicolor*, *Marasmius oreades*, *Pholiota aurivella*, *P. citrinopileatus*, and *Pleurotus cornucopiae*, was found either (*23*−*29*) (**Table 2**). Interestingly, a BLAST search of databases showed more than 85% homology between PCP-3A and tRNA-guanine-N(7)-methyltransferase from *Bacillus weihenstephanensis KBAB4* or dipeptidyl aminopeptidase from *Botryotinia fuckeliana B05.10* in N-terminal amino acid sequencing.

Growth Inhibition of U937 Cells by CM. There are two ways to evaluate the inhibitory effect of a natural product on leukemic cells, either by direct inhibition on the growth of these cells or by stimulating the secretion of differentiation-inducing factors from

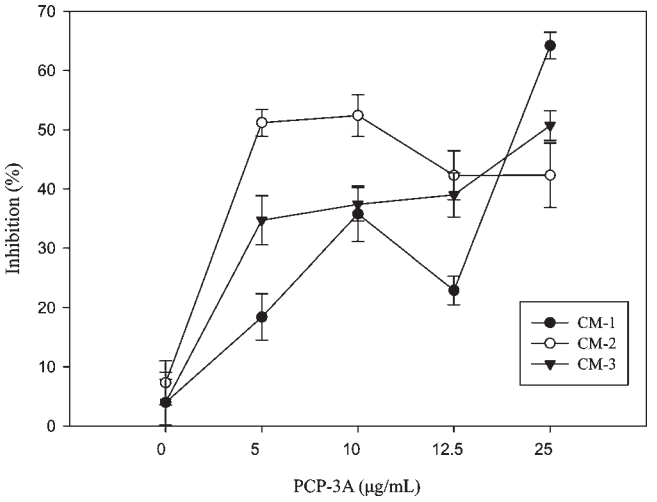

Figure 1. Indirect growth inhibitory effect of PCP-3A on U937 cells in the conditioned medium. U937 cells (1.25×10^5 cells/mL) were incubated for 3 days in the presence or absence (control) of 20% conditioned medium derived from treating MNC with PCP-3A for 1 (MNC-CM-1), 2 (MNC-CM-2), or 3 days (MNC-CM-3). Viable cells were counted after 3 days of cultivation. Data are the means of cell numbers ± SDs from three separate experiments.

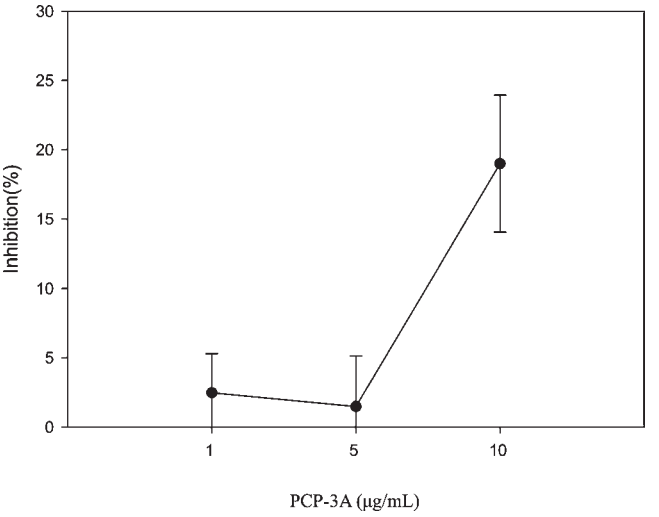

Figure 2. Direct growth inhibition of U937 cells (1.25×10^5 cells/mL) incubated with PCP-3A for 3 days. Data are the means of cell numbers ± SDs from three separate experiments.

immune cells to act on the tumor cells indirectly (*30*). The indirect method was usually used for observing the growth inhibition of tumor cells by the immunomodulatory components from a mushroom (*31*).

Figure 1 shows 17−64% growth inhibition on U937 cells in 3 days of incubation with the CM. Among the CM, MNC-CM-2 appeared to be the most potent one in inhibiting the growth of cells. The growth inhibition of MNC-CM-2 on tumor cells increased from 7.5 to 54% with the increase of PCP-3A concentration from 0 to 25 μg/mL, indicating dose dependency of the inhibitory effect. Growth inhibitions of U937 cells treated with the CM, which had been prepared with PCP-3A at 25 μg solids/mL, were 37−64% (**Figure 1**).

The direct effect from the residue of PCP-3A should be taken into consideration in assessing for the indirect growth inhibition effect from the experiment data. In the indirect experiment, the concentration of residual PCP-3A was diluted 5-fold from MNC-CM in constituting the medium for U937 incubation. Comparing the indirect and direct inhibition effects at the corresponding amounts (5 and 25 μg solids/mL in **Figure 1** as compared to 1 and 5 μg solids/mL in **Figure 2**, respectively), the indirect growth inhibition was much more effective than the direct inhibition. The indirect experiment was therefore subjected to little interference from the direct growth inhibition effect of PCP-3A.

The growth inhibitions of U937 cells in the treatment with the CM prepared with the cold water extract from *Hypsizigus marmoreus* at 200 μg/mL, with the semipurified extract from *F. velutipes* at 100 μg solids/mL and with the semipurified polysaccharides from *Antrodia camphorate* at 100 μg/mL, were reported to be 60, 51, and 55%, respectively (*6*, *32*). The growth inhibitory effect of PCP-3A appears no weaker than any of the above-mentioned mushroom extracts.

Effects of PCP-3A on Cytokine Production from Stimulated MNC. An approach to evaluate the immunomodulatory activity of an active component is to determine its capacity in influencing the cytokine production from MNC. The influence in cytokine production has been evaluated to prove the immunomodulatory activities of various fungal proteins, such as nonglycosylated proteins from mold *Aspergillus fumigatus* (*32*), glycoproteins from mushroom *Tricholoma matsutake* (*34*), and FIP-fve from mushroom *F. velutipes* (*25*).

There have been several indirect inhibition studies to demonstrate the relation between cytokine production and growth inhibition activity in MNC stimulated by various materials. For example, significant growth inhibition- and differentiation-inducing effects on human leukemia cells by MNC-CM prepared with *Ganoderma lucidum* (*31*), fu-ling (*Poria cocos*) (*35*), or colostrum (*36*) were found to be associated with the secretion of antitumor mediators, including cytokines interleukine (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, from MNC.

TNF-α is a cytokine produced by activated T- and B-lymphocytes, neutrophils, and NK cells (*37*) and is a potent tool for tumor cell necrosis (*38*). In the present study, PCP-3A was found to induce a significant increase in the production of TNF-α as compared to the control (**Figure 3A**). In addition, the TNF-α level in CM decreased with the increase in the duration of incubation with PCP-3A. The growth inhibitory effect of U937 cells also decreased with the decrease of TNF-α in the medium, indicating that the growth inhibition may be associated with the antitumor activity of TNF-α. IFN-γ is another important cytokine that inhibits the growth of tumor cells. In the IFN-γ levels of the cultured medium, stimulation of MNC with 5 or 10 μg/mL of PCP-3A for 1, 2, or 3 days significantly up-regulated the production of IFN-γ (**Figure 3B**). PCP-3A had no significant effect on the secretion of cytokine IL-4 from MNC (**Figure 3C**). Cytokine IL-2 was reported to induce endogenous lymphokine-activated killer activity in both normal and tumor-bearing mice and to offer a possible clinical application for immunotherapy against cancer without detrimental side effects (*39*). PCP-3A was able to induce a significantly increased production of IL-2 (pg/mL) as compared to the control (**Figure 3D**). The duration of incubation with PCP-3A did not affect the IL-2 level in CM. On the basis of the above-described experiment results, we propose that the antitumor effect of PCP-3A comes, at least partly, from its own immonomudulatory activity that involves the promotion in the release of cytokines TNF-α, IFN-γ, and IL-2 from MNC.

Effect of Antibody Neutralization on the Activity of PCP-3A-MNC-CM. To elucidate the role of cytokines released from PCP-3A-activated blood MNC on the growth inhibition of U937 cells, PCP-3A-MNC-CM-1 was neutralized with one or more cytokine-neutralizing antibodies before incubating with U937 cells. **Table 3** shows that the growth inhibitory potential of PCP-3A-MNC-CM on U937 cells dropped from 72.5 to 49.6 and 25.7% after treatment with anti-TNF-α and anti-IL-2, respectively. Anti-IFN-γ was not

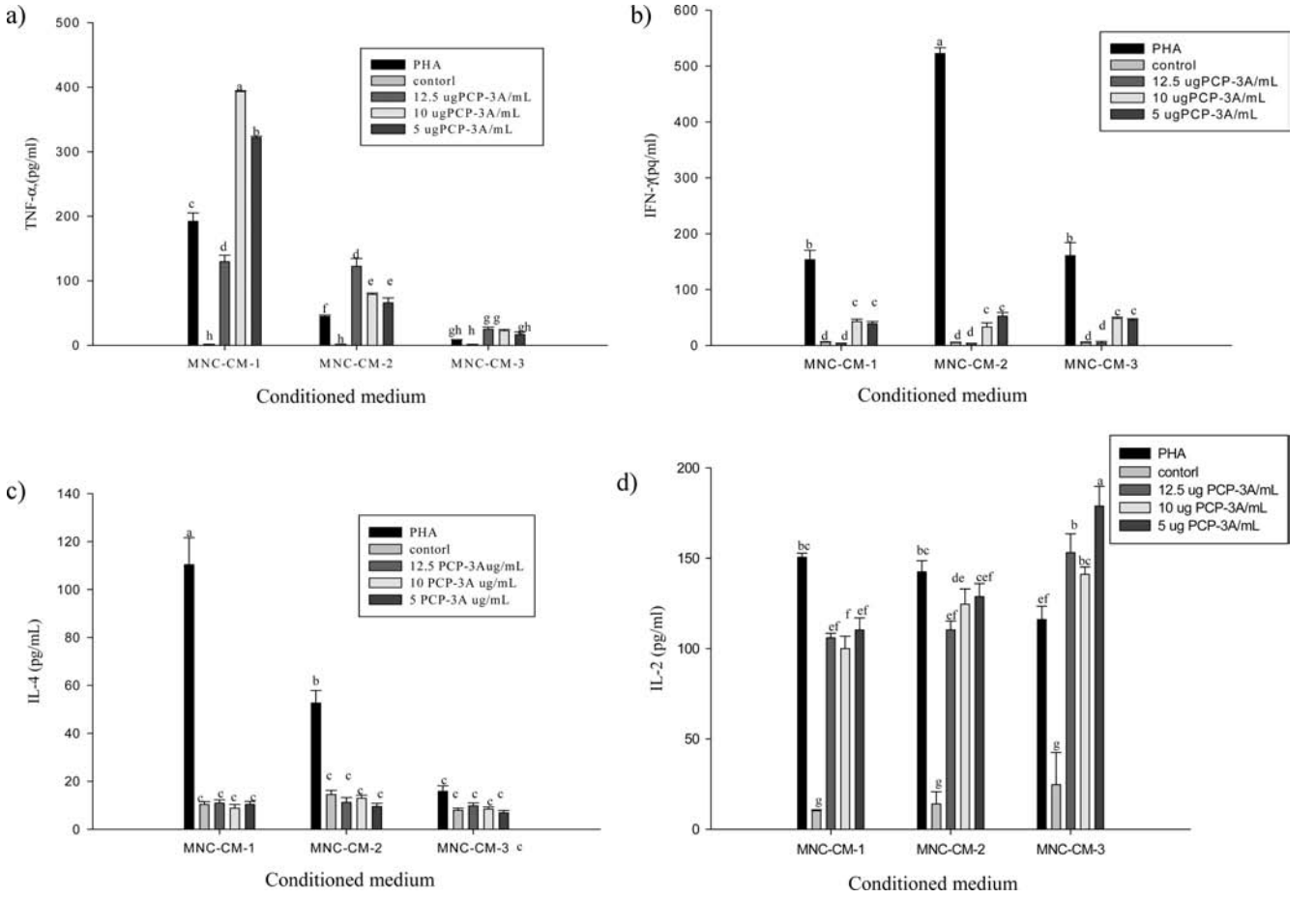

Figure 3. TNF-α (**A**), IFN-γ (**B**), IL-4 (**C**), and IL-2 (**D**) contents in the CM, which were prepared by stimulating MNC with various concentrations of PCP-3A for 1, 2, and 3 days. Cytokines were assayed with BioSource ELISA kit. The results from three replicated experiments are expressed as means ± SDs.

Table 3. Effect of Cytokine-Neutralizing Antibodies on the Growth Inhibition of U937 Cells Cultured with PCP-3A-MNC-CM1

medium	antibody	growth inhibition (%) of U937
PHA-MNC-CM-1 (positive control)		69.8 ± 3.3 a
PCP-3A-MNC-CM-1		72.5 ± 3.0 a
	normal rabbit serum	66.0 ± 3.3 a
	anti-TNF-α	49.6 ± 3.4 b
	anti-IFN-γ	72.3 ± 2.2 a
	anti-IL2	25.7 ± 1.5 d
	anti-TNF-α + IL2	9.4 ± 1.7 e
	anti-TNF-α + IFN-γ	34.6 ± 1.2 c
	anti-IFN-r + IL2	23.8 ± 3.0 d
	anti-TNF-α + IL2 + IFN-γ	6.2 ± 2.5 f

as effective as anti-TNF-α and anti-IL-2, but it exerted an enhancing effect to either one in reducing the growth inhibitory effect of PCP-3A-MNC-CM. The growth inhibition was further decreased to 6.2%, when the three antibodies were present together. The literature showed a similar effect that cytokines in MNC-CMs induced from *Cordyceps sinensis* (*31*) and *P. cocos* (*35*) could also inhibit the growth of U937 cells. The present antibody neutralization experiment showed that cytokines in MNC-CM play influential roles in the growth inhibition of U937 cells.

Effects of PCP-3A on Cytokine Production from Stimulated T Cells. $CD4^+$ T cells were separated from MNC and stimulated with PCP-3A. The cytokines released from T cells were then monitored to elucidate the succession among T-helper cells (Th cells). The effect of PCP-3A followed a trend to up-regulate the production of TNF-α (**Figure 4A**), IFN-γ (**Figure 4B**), and IL-2 (**Figure 4D**) and to down-regulate the production of IL-4 (**Figure 4C**) in the T cell-conditioned medium.

Immune responses are strongly influenced by the nature of the participating Th cells. Th cells are designated as Th 1 and Th 2 subpopulations based on their patterns of cytokine secretion. Th 1 cells, characterized by the production of IL-2, IFN-γ, and TNF-α, are mainly involved in cell-mediated immunity to intracellular infections (*4*, *7*, *10*). Th 2 cells, characterized by the production of IL-4, IL-5, and IL-10, are mainly involved in humoral immunity against extracellular infections (*4*, *18*). Mushroom compounds have been shown to induce polarization of Th population in one direction or the other. Some of these compounds promote Th 1 response, whereas others favor Th 2 response (*4*).

In the present study, PCP-3A was found to up-regulate the production of TNF-α, IFN-γ, and IL-2 from T cells stimulated with PCP-3A. On the other hand, the production of IL-4 was down-regulated. Meanwhile, PCP-3A stimulated T cell (**Figure 4**) to produce more cytokines than it did to MNC (**Figure 3**). Th cells are important regulators in the immune system (*40*). These results indicate that PCP-3A may induce a shift of T helper cells toward Th 1 response by activating the cell-mediated immunity against intracellular infections.

In conclusion, the present study showed that the N-terminal amino acid sequence of PCP-3A has no duplicates among all reported mushroom proteins and reconfirm that PCP-3A is a new mushroom protein. PCP-3A was found to stimulate the secretion of cytokines TNF-α, IL-2, and IFN-γ from MNC and $CD4^+$ T cell in

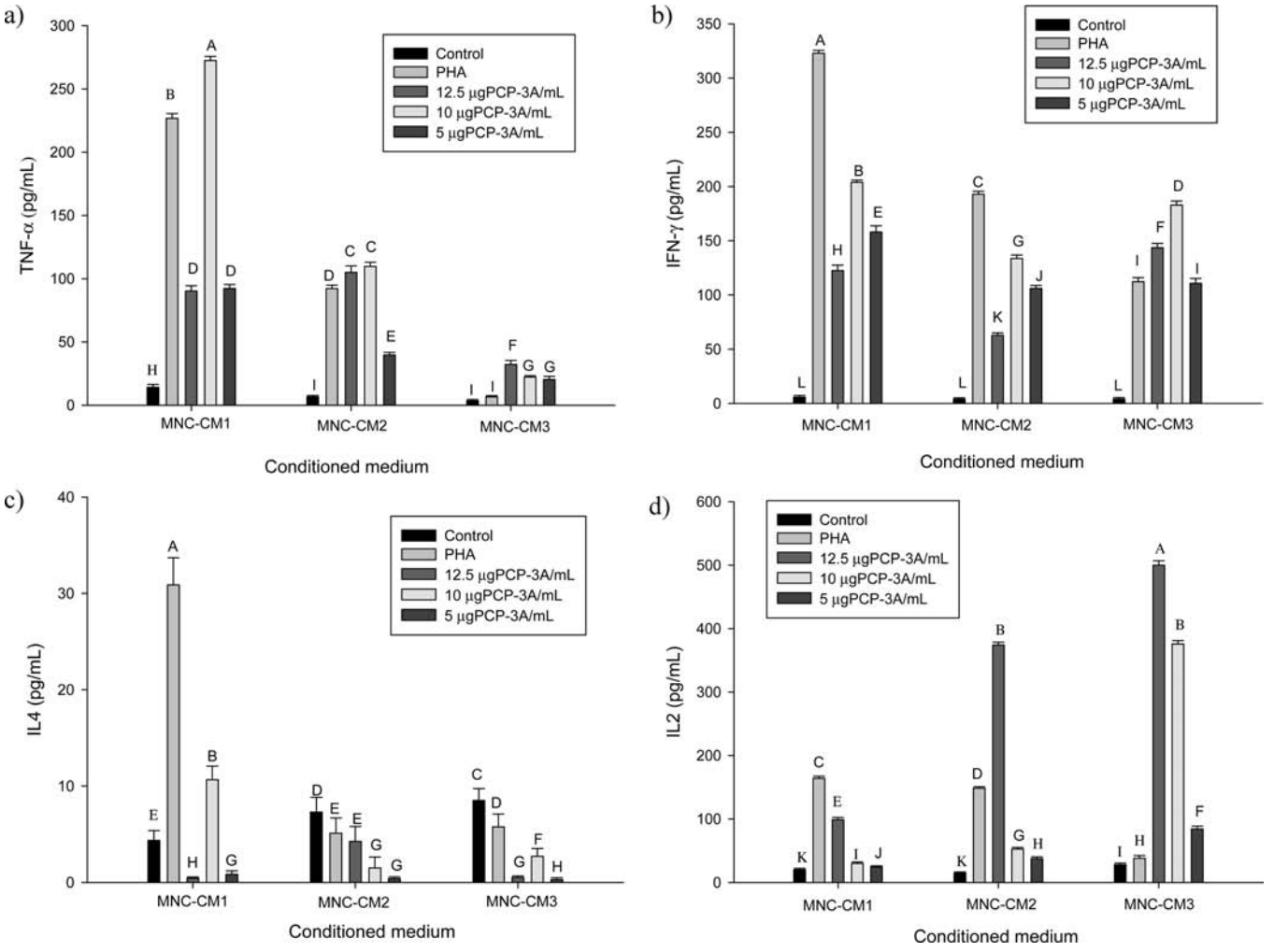

Figure 4. TNF-α (**A**), IFN-γ (**B**), IL-4 (**C**), and IL-2 (**D**) contents in the CM, which were prepared by stimulating $CD4^+$ T cells separated from MNC with various concentrations of PCP-3A for 1, 2, and 3 days. Cytokines were assayed with BioSource ELISA kit. The results from three replicated experiments are expressed as means ± SDs.

the indirect method that inhibited the growth of U937 cells. The above-described in vitro evidence supports the antitumor and immunomodulatory effects of PCP-3A. PCP-3A may be a possible material for developing into an antileukemia ingredient in health food. Further studies on these health effects of PCP-3A in vivo will be worthwhile.

ABBREVIATIONS USED

ANOVA, analysis of variance; CM, conditioned media; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; MNC, human peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PHA, phytohemeagglutinin; Th cells, T-helper cells.

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