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Potential Antitumor Activity of a Low-Molecular-Weight Protein Fraction from *Grifola frondosa* Through Enhancement of Cytokine Production

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ABSTRACT Edible mushrooms contain an abundance of immune-enhancing nutrients. Some of these compounds, referred to as biological response modifiers (BRMs), have been used in biological therapies for cancer treatment. We obtained a low-molecular-weight protein fraction (MLP-Fraction) from the fruiting body of the maitake mushroom *Grifola frondosa* by multiple sequential steps, including ethanol precipitation, DEAE-exchange chromatography, and gel filtration. The effect of the MLP-Fraction on the immune system was determined using normal mice. This resulted in a simultaneous increase in splenocyte proliferation and production of cytokines such as interleukin (IL)-1 α , tumor necrosis factor- α , IL-10, IL-12, and interferon (IFN)- γ . The expression levels of IFN- γ and IL-12 in antigen-presenting cells (APCs) and the activation of natural killer (NK) cells, macrophages, and dendritic cells were observed. These results suggest a mechanism in which NK cells are activated through cytokines produced by APCs. We also confirmed the possibility that the MLP-Fraction acts as a BRM using colon-26 carcinoma-bearing mice. This fraction enhanced the production of IL-12 and IFN- γ by splenocytes in tumor-bearing mice and clearly showed an inhibitory effect on tumor cell growth.

KEY WORDS: • antitumor activity • biological response modifiers • cytokine production • low-molecular-weight protein fraction

INTRODUCTION

BIOLOGICAL RESPONSE MODIFIERS (BRMs) are compounds typically used to treat diseases, related to disorders of the immune system (e.g., cancer, infection, allergies); BRMs augment or restore naturally occurring processes.^{1–4} They are also used to reduce certain side effects of some anticancer drugs such as mitomycin-C (MMC), 5-fluorouracil, and cisplatin.^{5–9} BRMs may also exhibit a direct or indirect anticancer effect by enhancing adaptive immunity associated with the activation of cytotoxic T-cells as well as innate immunity associated with the activation of macrophages, dendritic cells (DCs), and natural killer (NK) cells.^{10,11} Biological therapies such as BRMs have been used in cancer treatment with the aim of reducing the serious side effects of anticancer drugs.

Various mushroom extracts, categorized as BRMs, have been reported to possess antiviral, antibiotic, anti-inflammatory, antihypoglycemic, antihypocholesterolemic, and antihypotensive activities.^{11–17} Several polysaccharides extracted from mushrooms also possess antitumor and immunomodulatory properties. In 1987, we identified a poly-

saccharide consisting of a β -1,6 main chain with β -1,3 branches at a ratio of 5:4 from the edible maitake mushroom [*Grifola frondosa* (Dicks) Gray]. We named this polysaccharide “maitake D-Fraction” (MD-Fraction) because of the mixture of β -glucans (molecular weight, 1,000,000–1,200,000).^{18,19} Analysis of MD-Fraction revealed that its protein content was less than 0.1% of the total sugar weight.¹⁸ This indicated that MD-Fraction contained small proteins or peptides of unknown identity bound to β -glucan. MD-Fraction has been reported to enhance activation of macrophages, DCs, NK cells, and T-cells. Moreover, it exhibits antitumor activity, without side effects, in both tumor-bearing and normal mice.^{20–22} We also reported that combined treatment with MMC and MD-Fraction reduced the tumor size, compared to MMC treatment alone, by attenuating the decrease in the number of immune cells, such as macrophages, DCs, NK cells, and helper T-cells, as well as cytotoxic T-cells, in tumor-bearing mice.²³ In animal experiments, we demonstrated that MD-Fraction enhances the T-helper 1 (Th1)-dominant response, including cell-mediated immunity associated with cytotoxic T-cell activation, but not humoral immunity associated with B-cell activation.^{22,24} Phase II clinical trials evaluating MD-Fraction in breast cancer patients are underway at the Department of Integrative Medicine at the Memorial Sloan-Kettering Cancer Center, New York, NY, USA. In Japan, our research group monitored the activity of T-cells or

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NK cells during non-randomized trials investigating MD-Fraction therapy in patients with stage II–IV lung and breast cancer.^{25,26} In breast cancer, it has been reported that NK cell activity is closely related to disease progression.²⁶ Although most polysaccharides extracted from edible mushrooms have been reported to possess antitumor effects in animal experiments, their pharmacological use as anticancer drugs or immunomodulative drugs for cancer patients is not yet been approved, with the exception of lentinan from *Lentinus edodes* and polysaccharide-K (PSK) from *Coriolus versicolor*.^{27,28} To obtain approval, the most essential points to be clarified are the cellular and molecular mechanisms underlying their anticancer activity. MD-Fraction is difficult to purify because of its unstable structural conformation due to the frequent presence of high-molecular-weight side chains as well as the presence of contaminating lipopolysaccharide, a well-characterized bacterial cell wall polysaccharide with strong immunostimulating activities. We have therefore not been able to elucidate the intracellular signal transduction mechanism associated with the anticancer effect of MD-Fraction. However, elucidation of this mechanism is essential for enabling its pharmacological use in cancer patients.

Mushroom-derived products have a significant role in the discovery and development of potential medicinal agents. Water-soluble extracts and low-molecular-weight fractions from edible mushrooms have been the focus of research, and their immunostimulatory effects have been reported.²⁹ It has been demonstrated that GLPP (molecular weight, 6,600) from *Ganoderma lucidum* has the potential to be used as an adjuvant to conventional cancer treatment and in cancer prevention.³⁰ It was reported that the immunostimulating EX-GF-Fr. III from *G. frondosa* has a molecular weight of 2,800.³¹ The water-soluble extract from the maitake mushroom (*G. frondosa*) contains some pharmacological components also found in MD-Fraction. Small components consisting of sugars, small proteins, and peptides in water-soluble compounds might be derived from substances of high-molecular-weight material, including protein and sugar. This may be the result of enzymatic or physical destruction of larger structures during the process of obtaining the water-soluble extract from whole maitake mushrooms. We are therefore searching for fractions containing novel individual components of the MD-Fraction (new sugars, peptides, or small proteins; specifically those with low molecular weight) that enhance proliferation of immune cells and cytokine production and possess enhanced antitumor activity.

In the present study, we first used the original protocol based on the viability of the human monocyte cell line THP-1 to find fractions from the water-soluble extract of dried, powdered *G. frondosa* fruiting body. Second, to confirm the immunomodulating activity of this low-molecular-weight protein fraction (MLP-Fraction), we targeted cytokines (interleukin [IL]-1 α , IL-10, IL-12, interferon [IFN]- γ , and tumor necrosis factor [TNF]- α) produced by splenocytes, including macrophages, DCs, T-cells, and NK cells. We report that MLP-Fraction exhibited immunostimulating activity and enhanced the production of IL-12 and IFN- γ by splenocytes, resulting in an antitumor effect in mice. We

propose a useful protocol for easy identification of a potential fraction from numerous neutral components contained in water-soluble extracts.

MATERIALS AND METHODS

Animals

Female BALB/c mice (5 weeks old; Crea Japan Co., Tokyo, Japan) were raised for a week before use in experiments. Mice were fed a rodent diet, CE-2 (Crea Japan), and water was provided *ad libitum*. The study protocol was approved by the institutional ethics committees of Kobe Pharmaceutical University (Kobe, Japan).

Preparation of Hot extract and 5000cut-Fraction from *G. frondosa* fruiting body

Dried powder from the fruiting body of the edible maitake mushroom (*G. frondosa*) was kindly provided by Yukiguni Maitake Co. (Niigata, Japan). The dried powder was suspended in deionized water and autoclaved for 15 minutes at 110°C to obtain a hot extract designated "Hot extract." A 5000cut-Fraction was filtered from the Hot extract by centrifugation to obtain substances having a molecular weight less than 5,000.

Fractionation of MLP-Fraction

Hot extract was prepared as described previously. Ethanol (EtOH) was added at a final concentration of 50% to the prepared Hot extract at 4°C. The precipitate was removed, and the remaining mixture was allowed to stand for an additional 2 hours to allow further precipitation. The supernatant was obtained by centrifugation at 7,000 g and named "50% EtOH sup." The supernatant was applied to a DEAE-Cellulofine (Seikagaku Co., Tokyo) column to remove nonadherent substances using Tris-HCl buffer (pH 8.0) as the eluant. Adherent substances were then eluted with 0.2 M NaCl in Tris-HCl buffer (pH 8.0). Each fraction was tested for its effect on the proliferative activity of MMC-treated THP-1 cells. The peak fractions showing maximum activity were collected (Fig. 1A). The mixture obtained was designated "DEAE-Fraction," which was then separated by G-10 gel filtration using Milli-Q[®] (Millipore, Bedford, MA, USA) water to obtain the crude MLP-Fraction (Fig. 1B). A second more intense gel filtration process was performed to further purify the crude MLP-Fraction and to remove salts (Fig. 1C). The fraction in which the protein peak coincided with peak activity was then collected, filtered, and stored at 4°C. The fraction was named "MLP-Fraction." The protein yield of MLP-Fraction from Hot extract was 0.01% (Table 1). MLP-Fraction contained a protein concentration of 1.5 μ g/mL but no sugar.

Cells

THP-1 (a human monocyte cell line) and colon-26 carcinoma cells (a mouse rectum carcinoma cells) were provided by the Cell Resource Center for Biomedical Research

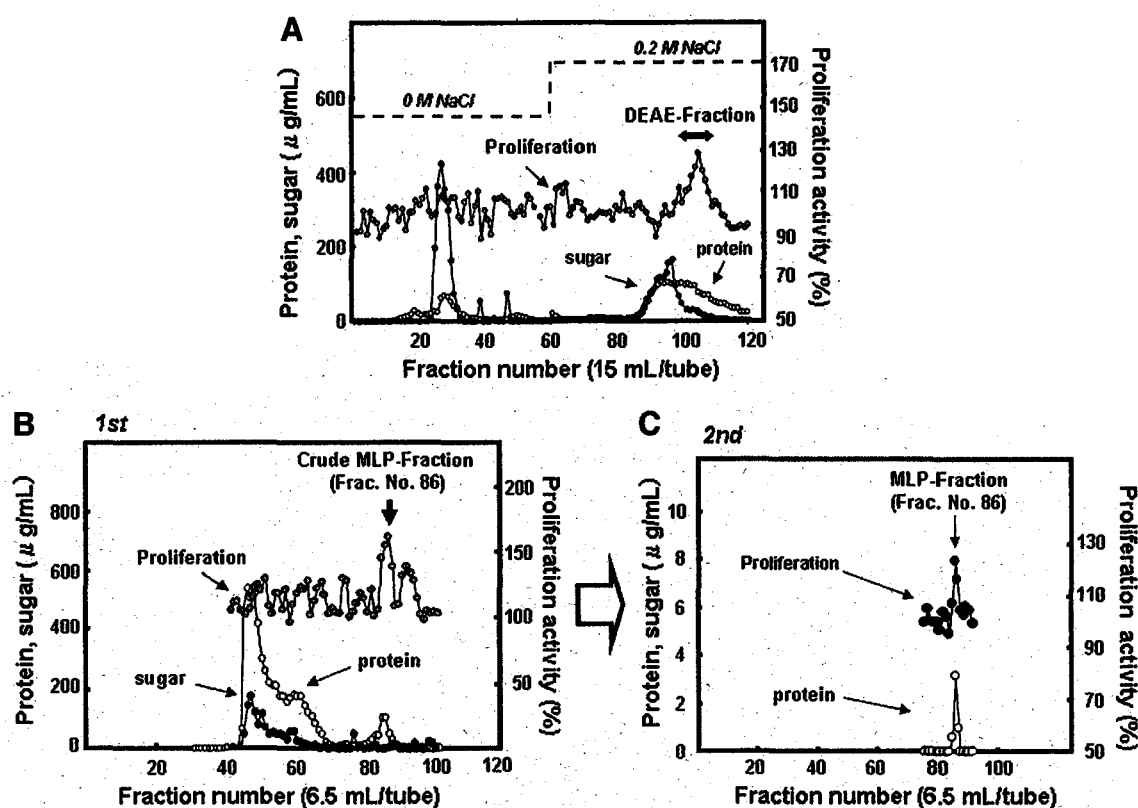


FIG. 1. Pattern of DEAE-exchange chromatography of (A) Hot extract and Sephadex G-10 filtration of DEAE-Fraction ([B] crude material and [C] purified). The 50% EtOH sup was applied to a DEAE-Cellulofine column (3 cm diameter \times 60 cm). Nonadherent substances were removed with 12.5 mM Tris-HCl (pH 8.0), and the DEAE-Fraction was then eluted with 0.2 M NaCl/12.5 mM Tris-HCl buffer (pH 8.0) and collected. DEAE-Fraction (A) and crude MLP-Fraction (fraction 86) (B) were applied to a Sephadex G-10 column (2 cm diameter \times 90 cm) and eluted with Milli-Q water (C). The total amounts of sugar and protein contained in each fraction were determined and then examined using 10 μ M MMC for 15 hours to determine proliferation activity (percentage).

at Tohoku University (Sendai, Japan). THP-1 cells and colon-26 carcinoma cells were cultured in RPMI-1640 medium (Nissui Seiyaku Co., Tokyo) containing penicillin/streptomycin and 10% inactivated fetal bovine serum and maintained at 37°C in a 5% CO₂ incubator.

Antibodies

Antibodies were purchased from Pharmingen Co. (San Diego, CA, USA). The antibodies were: anti-mouse CD16/CD32 (2.4G2) (0.5 mg/mL), R-phycoerythrin (PE)- or fluo-

rescein isothiocyanate (FITC)-conjugated anti-mouse CD69 (H1.2F3) (0.5 mg/mL or 0.2 mg/mL), FITC-conjugated anti-mouse NK1.1 (PK136) (0.5 mg/mL), R-PE-conjugated anti-mouse CD49b/PanNK (DX5) (0.2 mg/mL), BD-Cy-Chrome (PE-CyTM5)-conjugated anti-mouse CD3 ϵ (145-2C11) (0.1 mg/mL), FITC-conjugated anti-mouse I-A/I-E (MHC II) (2G9) (0.5 mg/mL), PE-Cy5-conjugated anti-mouse CD45R/B220 (RA3-6B2) (0.1 mg/mL), PE-Cy7-conjugated anti-mouse CD11b (M1/70) (0.1 mg/mL), FITC-conjugated anti-mouse CD11c (HL3) (0.5 mg/mL), R-PE-conjugated anti-mouse IFN- γ XMG1.2 (0.1 mg/mL),

TABLE 1. COMPARISON OF THE EFFECTS OF HOT EXTRACT, 50% ETOH SUP, DEAE-FRACTION, CRUDE MLP-FRACTION, AND MLP-FRACTION ON THP-1 CELL PROLIFERATION ACTIVITY IN THE PRESENCE OF MMC

Sample	Protein concentration (μ g/mL)	Total protein (mg)	Yield of protein (%)	Proliferation activity ^a
Hot extract	6,615.5	132.310	100.00	100.5 \pm 2.23
50% EtOH sup	5,814.5	116.290	88.90	100.1 \pm 1.36
DEAE-Fraction	92.6	12.506	9.47	100.3 \pm 2.03
Crude MLP-Fraction	27.7	0.224	0.17	101.4 \pm 0.09
MLP-Fraction	1.5	0.015	0.01	124.5 \pm 8.64*
Control	0.0	0.000	—	100.0 \pm 0.00

^aEach sample was prepared (1.5 μ g/mL protein), and 1 μ L of the prepared sample or Milli-Q water (control) was checked using 10 μ M MMC for 15 hours to determine proliferation activity (percentage). Dunnett's test was used for statistical comparisons.

* P < .05, in comparison to the control with MMC.

and R-PE-conjugated anti-mouse IL-12 (p40/p70) C15.6 (0.1 mg/mL).

Evaluation of tumor inhibition ratio (TIR)

The colon-26 carcinoma cell strain originated from a BALB/c female mouse. We therefore established a colon-26 carcinoma-bearing mouse by transplantation of carcinoma cells into a female mouse. Colon-26 carcinoma cells (1×10^5) were implanted into the right axillary region of female BALB/c mice (6 weeks old). After 24 hours, MLP-Fraction (1.5, 7.5, or 15.0 ng per mouse per day) or saline was administered intraperitoneally to the carcinoma-bearing mice for 7 consecutive days. On day 8, the tumor was excised and weighed to obtain the TIR. TIR was calculated according to the following formula

$$\text{TIR (\%)} = (1 - [\text{mean solid tumor weight of MLP-treated group} / \text{mean solid tumor weight of saline-treated group}]) \times 100$$

Preparation of splenocytes

MLP-Fraction (1.5, 7.5, or 15.0 ng per mouse per day) or saline was administered intraperitoneally to separate groups, consisting of four to six normal female BALB/c mice or colon-26 carcinoma-bearing mice, for 7 consecutive days. On day 8, spleens were removed, and cells were prepared (1×10^6 cells/0.1 mL per 96-well plate) and tested for cytokine production using enzyme-linked immunosorbent assay kits (Pierce Biotechnology Inc., Rockford, IL, USA) after a 24-hour incubation. Splenocytes were also used to test for the activation of macrophages, DCs, and NK cells and the intracellular expression of IL-12 and IFN- γ in splenic antigen-presenting cells (APCs), as well as for their proliferation activity.

Evaluation of cell viability and proliferation activity of THP-1 cells

To determine THP-1 cell viability, each test sample was added to THP-1 cells (4.8×10^4 /0.1 mL per 96-well plate) and incubated with and without 10 μ M MMC for 15 hours at 37°C in a 5% CO₂ incubator. After incubation, 10 μ L of WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] reagent (Nacalai Tesque Co., Kyoto, Japan) was added to the cultured cells and incubated for an additional 2 hours. Absorbance was measured at 450 nm (reference, 650 nm) to determine cell viability and proliferation activity by measuring intracellular dehydrogenase activity. Sterilized Milli-Q water was used as the control. Cell viability (percentage) and proliferation activity (percentage) of THP-1 cells were calculated according to the following formula:

$$\text{Viability (\%)} = (\text{Absorbance of each fraction sample with MMC} / \text{absorbance of control without MMC}) \times 100$$

$$\text{Proliferation activity (\%)} = (\text{Absorbance of each fraction sample with MMC} / \text{absorbance of control with MMC}) \times 100$$

To determine splenocyte proliferation, MLP-Fraction was administered to normal mice for 7 consecutive days. Spleens were then removed, and the prepared splenocytes (1×10^6 /0.1 mL per 96-well plate) were incubated with WST-8 reagent for 2 hours, and absorbance (450 nm/reference 650 nm) was determined. Saline was used as the control. Proliferation activity (percentage) was calculated according to the following formula:

$$\text{Proliferation activity (\%)} = (\text{Absorbance of each sample from MLP-Fraction-treated mice} / \text{absorbance of sample from saline-treated mice}) \times 100$$

Flow cytometry

Splenic macrophages, DCs, APCs, and NK cells were stained for specific cell surface markers: macrophages (CD11b⁺), DCs (CD11c⁺), APCs (MHC II⁺ B220⁻), and NK cells (CD3 ϵ ⁻PanNK⁺ or CD3 ϵ ⁻NK1.1⁺). Macrophages, DCs, and NK cells were subsequently double-stained for CD69 as the earliest activation marker of these cells.^{20,32,33} In brief, for cell surface antigen detection in NK cells, 100 μ L of splenocytes (1×10^7 /mL) was mixed with 0.5 μ L of anti-mouse CD16/CD32 in a tube to block the Fc receptor and allowed to react at 4°C for 5 minutes. The mixture was incubated with 0.5 μ L of FITC- or 1.25 μ L of R-PE-conjugated anti-mouse CD69 (0.5 mg/mL), 1.25 μ L of R-PE-conjugated anti-mouse PanNK or 0.5 μ L of FITC-conjugated anti-mouse NK1.1, and 1.25 μ L of PE-Cy5-conjugated anti-mouse CD3 ϵ (mouse antibodies $\geq 1 \mu$ g) at 4°C for 30 minutes, washed with washing solution (0.09% NaN₃ and 1% fetal bovine serum in phosphate-buffered saline), then suspended in 0.5 mL of washing solution, and enumerated using a FACScan[™] flow cytometer (Beckton Dickinson, Grenoble, France). For cell surface antigen detection of macrophages and DCs, 1.25 μ L of PE-Cy7-conjugated anti-mouse CD11b (for macrophages), 0.5 μ L of FITC-conjugated anti-mouse CD11c (for DCs), and 1.25 μ L of R-PE-conjugated anti-mouse CD69 (for macrophages and DCs) were used, respectively.

For detection of APC intracellular IL-12 or IFN- γ , 2-mL aliquots of splenocytes (2×10^6 /mL) were applied to 24-well plates. Ionomycin (1 μ g/mL) and phorbol 12-myristate-13-acetate (25 ng/mL) were added to each well and subsequently incubated with 2.8 μ L of Golgi Stop[™] (Pharmingen Co.) at 37°C in an atmosphere of 5% CO₂ for 4 hours. After incubation, 0.5 μ L of anti-mouse CD16/CD32 was mixed with the cells and allowed to react at 4°C for 5 minutes. Subsequently, 1.25 μ L of FITC-conjugated

anti-mouse MHC II and 1.25 μ L of PE-Cy5-conjugated anti-mouse CD45R/B220 were added to the cells and incubated at 4°C for 20 minutes. After the reaction, the cells were washed with staining medium (0.05% NaN₃ and 1% fetal bovine serum in phosphate-buffered saline), incubated with 100 μ L of Cytofix/Cytoperm™ (Pharmingen Co.) at 4°C for 20 minutes, and washed again with Perm/Wash (Pharmingen Co.). The stained cells were reacted with 2.5 μ L of R-PE-conjugated anti-mouse IL-12 or anti-mouse IFN- γ at 4°C for 30 minutes, washed with Perm/Wash, suspended in 50 μ L of staining medium, and enumerated using FACScan flow cytometry.

Determination of concentrations of IL-1 α , IL-10, IL-12, TNF- α , and IFN- γ

Splenocytes (1×10^7 /mL per 12-well plate) were cultured for 24 hours at 37°C in a 5% CO₂ incubator. The culture supernatant was collected by centrifugation. The concentrations of IL-1 α , IL-10, IL-12, TNF- α , and IFN- γ were determined using the appropriate mouse cytokine enzyme-linked immunosorbent assay kit (Pierce Biotechnology Inc.).

Determination of the concentration of sugars and proteins

Total sugar concentration was determined by the anthrone method.³⁴ Total protein concentration was determined using a micro BCA protein assay kit (Pierce Biotechnology Inc.).

Statistical analysis

Data are expressed as mean \pm SD values. Significant differences were analyzed using the analysis of variance and a *post hoc* test (Dunnett's test). A value of $P < .05$ was considered statistically significant between each sample (Hot extract, 50% EtOH sup, DEAE-Fraction, crude MLP-Fraction, or MLP-Fraction)-treated group and the control group. Student's *t* test was used for comparisons between two groups. A value of $P < .01$ or $P < .001$ was considered statistically significant.

RESULTS

Effects of Hot extract and 5000cut-Fraction on viability of MMC-treated THP-1 cells

Figure 2 shows the effects of Hot extract and 5000cut-Fraction on the viability of MMC-treated THP-1 cells. MMC treatment alone decreased the viability to approximately 40% of the control. Viability increased in cells treated with Hot extract and 5000cut-Fraction in a concentration-dependent manner. Addition of Hot extract or 5000cut-Fraction alone had no effect on cell viability compared with the control without MMC (data not shown). We also examined the cytotoxicity of Hot extract or 5000cut-Fraction towards THP-1 cells using trypan blue; no toxicity to THP-1 cells was observed. These results suggest the existence of promising low-molecular-weight products in Hot extract and 5000cut-Fraction.

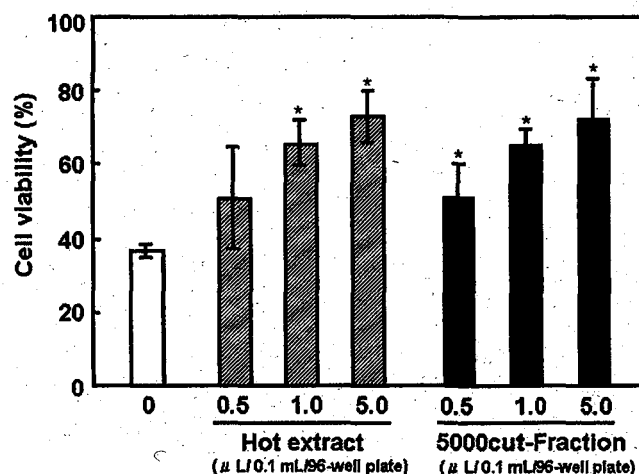


FIG. 2. Effects of Hot extract and 5000cut-Fraction on viability of MMC-treated THP-1 cells. The effects of Hot extract and 5000cut-Fraction on THP-1 cells were investigated using 10 μ M MMC for 15 hours to determine viability (percentage). Data are mean \pm SD values of three independent experiments ($n = 3-5$ per experiment). Statistical significance was evaluated by analysis of variance and Dunnett's test. * $P < .05$, in comparison to the group without addition of Hot extract and 5000cut-Fraction.

Effects of MLP-Fraction on viability and proliferation activity of MMC-treated THP-1 cells

To evaluate the proliferation activity of MLP-Fraction, we tested 1 μ L of each sample in a 96-well plate. The results were as follows: Hot Extract, $133.8 \pm 8.62\%$ (6,615.5 ng of protein); 50% EtOH sup, $157.6 \pm 4.93\%$ (5,814.5 ng of protein); DEAE-Fraction, $131.1 \pm 12.05\%$ (92.6 ng of protein); crude MLP-Fraction, $149.2 \pm 2.93\%$ (27.7 ng of protein); MLP-Fraction, $124.5 \pm 8.62\%$ (1.5 ng of protein). To compare differences in activity between Hot extract and MLP-Fraction, Hot extract and MLP-Fraction were individually added to the plates at identical protein concentrations (Table 1). When MMC-treated THP-1 cells were stimulated with Hot extract (1.5 ng/0.1 mL per well) in a 96-well plate, no change was observed in proliferation activity compared with the MMC-treated control. In contrast, MLP-Fraction significantly increased the activity to 1.25 times that of the control. The results indicate that even though MMC reduced THP-1 cell proliferation activity, MLP-Fraction acted to overcome these adverse effects, by increasing proliferation activity. We compared the effect of MLP-Fraction on THP-1 cell viability in the presence and absence of MMC to determine whether MLP-Fraction had an effect on the activity in the absence of MMC. Figure 3 shows that THP-1 cell viability increased in a concentration-dependent manner when THP-1 cells were stimulated with MLP-Fraction, in both the presence and absence of MMC. This suggested that MLP-Fraction could act as a BRM in the absence of MMC by stimulating the immune system in mice.

Proliferation activity of splenocytes from normal mice administered MLP-Fraction

To investigate whether MLP-Fraction increases splenocyte proliferation, MLP-Fraction was administered to normal

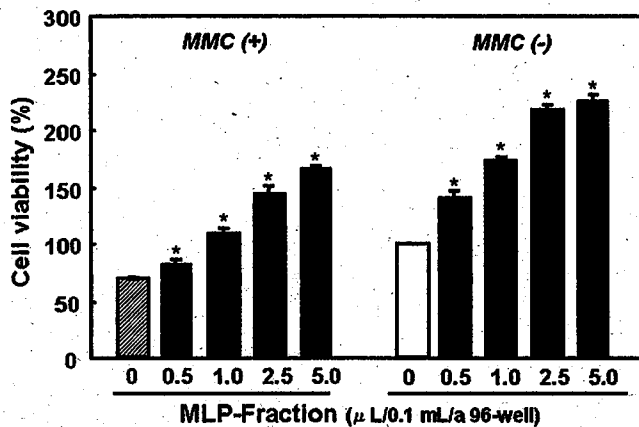


FIG. 3. Comparison of the effects of MLP-Fraction on THP-1 cell viability in the presence and absence of MMC. The effect of MLP-Fraction on THP-1 cells was evaluated in the presence and absence of 10 μ M MMC for 15 hours to determine viability (percentage). Values are mean \pm SD values of three independent experiments ($n=3-5$ per experiment). Statistical significance was evaluated by analysis of variance and Dunnett's test. * $P < .05$, in comparison with the control with and without MMC, individually.

BALB/c mice for 7 consecutive days in the absence of MMC. The activity was significantly increased in splenocytes from MLP-Fraction-treated mice compared with those from saline-treated mice, with maximum activity observed at a dosage of 7.5 ng (Fig. 4). These results suggest that MLP-Fraction stimulated the proliferation of splenocytes in normal mice, indicating that MLP-Fraction may enhance the immune system through the activation of macrophages, DCs, and NK cells.

Production of IL-1 α , IL-10, IL-12, TNF- α , and IFN- γ by splenocytes from normal mice administered MLP-Fraction

We measured the levels of IL-1 α , IL-10, IL-12, TNF- α , and IFN- γ produced by splenocytes from normal mice after administration of MLP-Fraction for 7 consecutive days. Figure 5 shows that treatment with MLP-Fraction increased the levels of IL-10, IL-12, TNF- α , and IFN- γ in a dose-dependent manner. The IL-1 α level was maximal at a dosage of 7.5 ng. The results support the hypothesis that MLP-Fraction directly or indirectly enhances the activation of the macrophages, DCs, APCs, and NK cells that are responsible for producing these cytokines.

Activation of splenic macrophages, DCs, and NK cells in normal mice administered MLP-Fraction

We investigated the activation of splenic macrophages, DCs, and NK cells by detecting the expression ratio (percentage) of CD69 (a known early activation marker of these immune cells) (Fig. 6). Treatment with MLP-Fraction increased the expression ratio of CD69 on macrophages and DCs at a dosage between 1.5 and 15 ng (Fig. 6A and B). The CD69 expression ratio on NK cells increased in a dose-dependent manner (Fig. 6C and D). In particular, activation of NK cells was markedly increased compared with that of

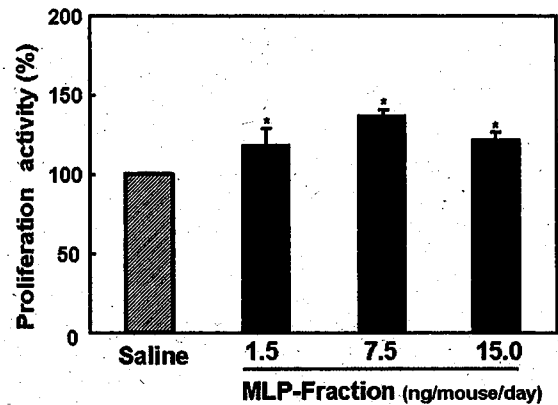


FIG. 4. Effect of MLP-Fraction on splenocyte proliferation in normal mice. MLP-Fraction was administered (intraperitoneally) to four separate groups consisting of four to six normal BALB/c mice (6 weeks old) for 7 consecutive days. Splenocytes were collected on day 8, and the effects of MLP-Fraction on splenocyte proliferation activity were determined. Data are mean \pm SD values of three independent experiments ($n=3-5$ per experiment). Statistical significance was evaluated by analysis of variance and Dunnett's test. * $P < .05$, in comparison with the saline-treated group.

the other cell types. These results suggest that NK cells were activated through IFN- γ and IL-12, produced by splenic macrophages and DCs, in mice treated with MLP-Fraction.

Expression of intracellular IFN- γ and IL-12 in splenic APCs from normal mice administered MLP-Fraction

NK cells were activated by MLP-Fraction (Fig. 6C and D), but IFN- γ expression in splenic NK cells did not differ significantly between MLP-treated and control mice (data not shown). This result suggests that the increase in IFN- γ produced by splenocytes was derived from APCs, including activated macrophages and DCs. Therefore, we targeted macrophages and DCs and analyzed the expression (percentage) of these cytokines in APCs depleted of B-cells (MHC II⁺B220⁻ cells in a monocyte gate), as detected by flow cytometry. The expression ratio of IFN- γ and IL-12 in APCs cells increased significantly after MLP-Fraction administration (7.5 ng per mouse per day) compared with saline administration (Fig. 7). This suggests that MLP-Fraction activated NK cells through production of IFN- γ and IL-12 by activated macrophages and DCs.

Growth of colon-26 carcinoma cells in mice administered MLP-Fraction

To investigate its antitumor effect, MLP-Fraction was administered to colon-26 carcinoma-bearing mice for 7 consecutive days. As expected, MLP-Fraction inhibited carcinoma cell growth; the TIR was 67% at a dosage of 7.5 ng (Fig. 8). This result indicated that MLP-Fraction has an antitumor effect.

Production of IFN- γ and IL-12 by splenocytes in colon-26 carcinoma-bearing mice administered MLP-Fraction

We measured levels of IFN- γ and IL-12 produced by splenocytes from carcinoma-bearing mice administered an

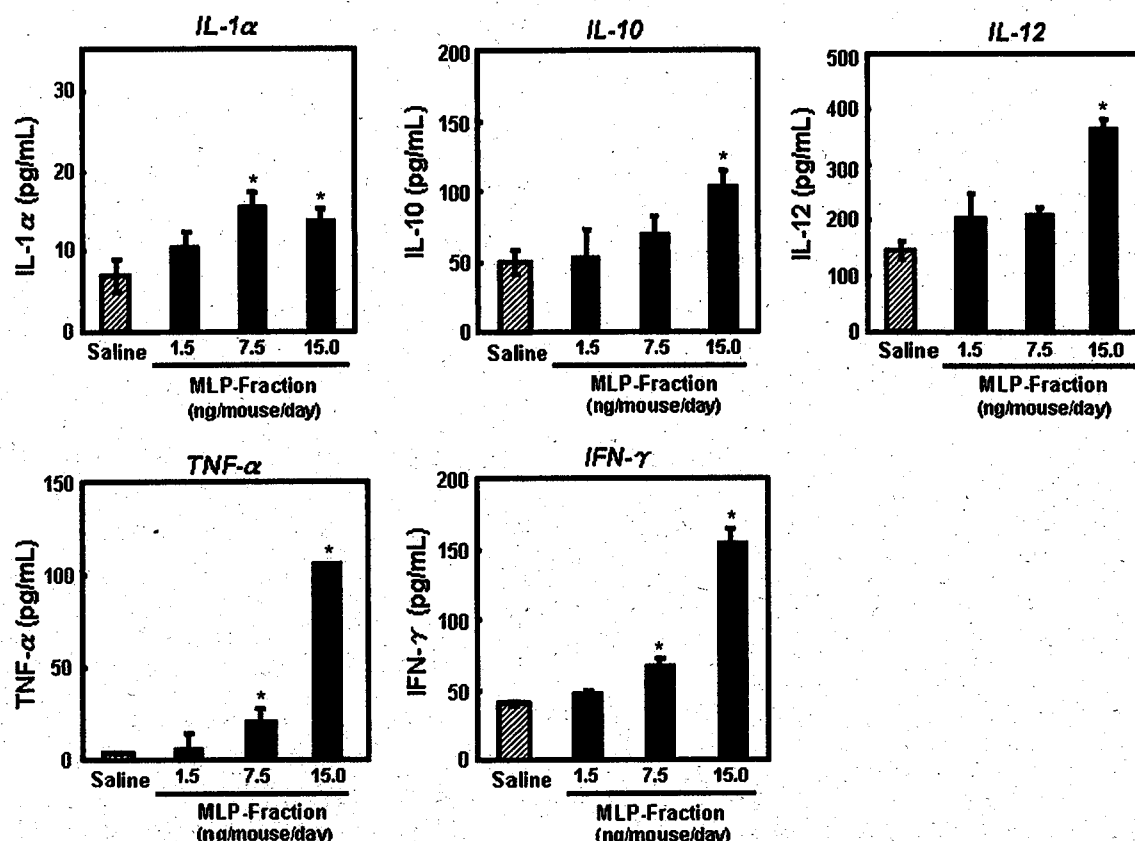


FIG. 5. Effects of MLP-Fraction on production of IL-1 α , IL-10, IL-12, TNF- α , and IFN- γ by splenocytes from normal mice. MLP-Fraction was administered (intraperitoneally) to four separate groups consisting of four to six normal BALB/c mice (6 weeks old) for 7 consecutive days. Splenocytes were collected on day 8, and the effects of MLP-Fraction on production of IL-1 α , IL-10, IL-12, TNF- α , and IFN- γ by splenocytes were determined after a 24-hour incubation. Data are mean \pm SD values of two or three independent experiments ($n=3$ per experiment). Statistical significance was evaluated by analysis of variance and Dunnett's test. * $P < .05$, in comparison with the saline-treated group.

MLP-Fraction at a dosage of 7.5 ng. There were significant differences between the MLP-Fraction-treated group and the saline-treated group on day 8 (Fig. 9). This result suggests that MLP-Fraction enhanced the activation of macrophages, DCs, and NK cells, thereby inhibiting the growth of tumor cells.

DISCUSSION

The immune system has a crucial role in body defense and the prevention of cancer. Besides cytokines such as TNF- α , IFN, ILs, and growth factors, many polysaccharides have been reported to activate the immune system, thereby by enhancing host defenses. Therefore, polysaccharides are expected to be useful in the treatment and prevention of cancer. The polysaccharides PSK and lentinan extracted from edible mushrooms are used to treat cancer patients.^{27,28} We discovered MD-Fraction from the maitake mushroom and studied its antitumor activity *in vivo*¹⁸⁻²³ and *ex vivo*.^{25,26} MD-Fraction was reported to activate macrophages and DCs and thereby induce a Th1-dominant response associated with cytotoxic T-cells. Moreover, MD-Fraction indirectly activated NK cells through production of IL-12 and IFN- γ by APCs, including macrophages and DCs,

in normal and tumor-bearing mice. It has been reported that the activation of NK cells significantly contributes to tumor size reduction in animals and humans. Although it has been reported that these polysaccharides enhance the immune system in mice, *in vitro* cellular and molecular experiments have yet to elucidate their mechanisms of anticancer activity.

Recently, researchers have focused not only on mushroom products having high molecular weights, but also on those having low molecular weights. There are many types of useful products in mushroom extract, such as sugars, proteins, peptides, amino acids, and nucleic acids. Reports on anticancer peptides indicate that they stimulate the immune system and can be used as BRMs in cancer patient.³⁵⁻³⁷ Some peptides are also known to stimulate the innate immune response in living organisms.³⁸ Many cationic and anionic peptides have been described as immunostimulating agents, such as antimicrobial peptides.

We found that MD-Fraction increased the number of splenocytes in combination treatment with MMC.²³ The result clearly indicated that treatment with MD-Fraction reversed the decrease in splenocyte numbers induced by MMC to normal levels without changing the ratio of only APCs, helper T-cells, and cytotoxic T-cells in splenocytes.

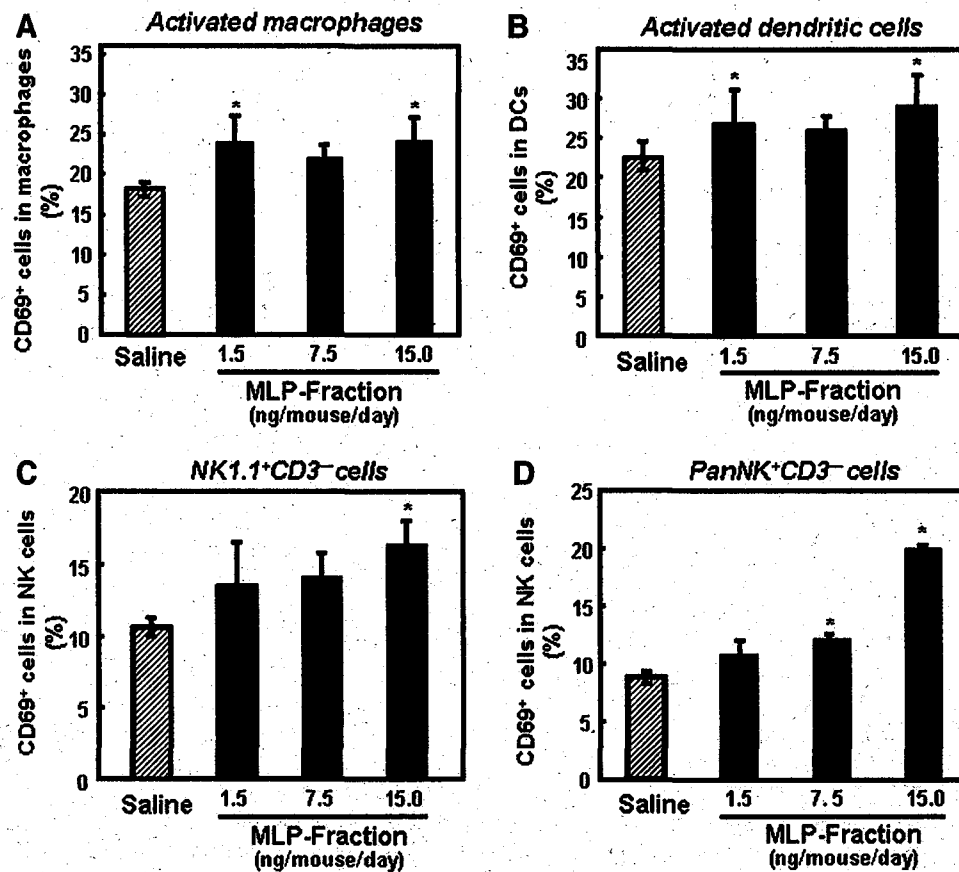


FIG. 6. Effects of MLP-Fraction on activation of splenic macrophages, DCs, and NK cells. MLP-Fraction was administered (intraperitoneally) to four separate groups consisting of four to six normal BALB/c mice (6 weeks old) for 7 consecutive days. Splenocytes were collected on day 8, and the effects of MLP-Fraction on the activation of (A) splenic macrophages (CD11b⁺ cells in a monocyte gate), (B) splenic DCs (CD11c⁺ cells in a monocyte gate), and splenic NK cells [(C) CD3 ϵ ⁻NK1.1⁺ cells or (D) CD3 ϵ ⁻PanNK⁺ cells in a lymphocyte gate) were determined by flow cytometry. The percentage of CD69⁺ cells in each cell represents the degree of cell activation. Data are mean \pm SD values of two or three independent experiments ($n=3$ per experiment). Statistical significance was evaluated by analysis of variance and Dunnett's test. * $P < .05$, in comparison with the saline-treated group.

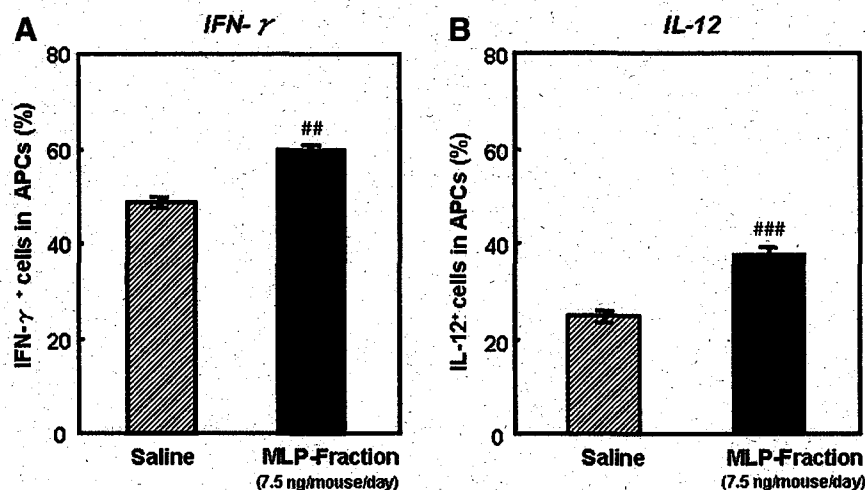


FIG. 7. Effects of MLP-Fraction on expression of intracellular (A) IFN- γ and (B) IL-12 in splenic APCs. MLP-Fraction was administered (intraperitoneally) to two separate groups consisting of four to six normal BALB/c mice (6 weeks old) for 7 consecutive days. Splenocytes were collected on day 8, and the effect of MLP-Fraction on the percentage of IFN- γ - or IL-12-expressing cells in splenic APCs (MHCII⁺B220⁻ cells in a monocyte gate) was determined by flow cytometry. Data are mean \pm SD values of two or three independent experiments ($n=3$ per experiment). Significance of differences was tested using Student's t test. ^{##} $P < .01$, ^{###} $P < .001$, in comparison with the saline-treated group.

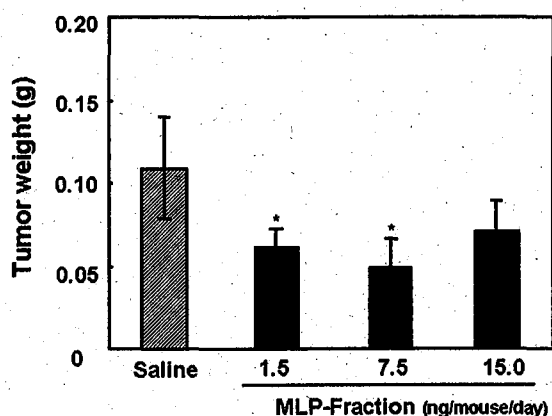


FIG. 8. Effect of MLP-Fraction on colon-26 carcinoma cell growth in mice. MLP-Fraction was administered (intraperitoneally) to four separate groups consisting of six colon-26 carcinoma-bearing BALB/c mice (6 weeks old) for 7 consecutive days, and the effect of MLP-Fraction on carcinoma cell growth was determined by measuring solid tumor weight. Data are mean \pm SD values of three independent experiments. Statistical significance was evaluated by analysis of variance and Dunnett's test. * $P < .05$, in comparison with the saline-treated mice.

This result demonstrated that the expression of the antitumor effect of MD-Fraction corresponded to the recovery of immune cell proliferation. Therefore, we developed an original protocol to screen for low-molecular-weight fractions from water-soluble extracts that increase THP-1 cell proliferation. Using this protocol, we attempted to find a small protein or peptide fraction (MLP-Fraction) and to investigate its antitumor effect.

MLP-Fraction increased the viability of MMC-treated THP-1 cells; regardless of the presence or absence of MMC

(Fig. 3). MLP-Fraction did not contain sugar and was detected by ultraviolet adsorption when the APC assay showed peptide binding in the MLP-Fraction. The elution pattern from anion exchange chromatography showed the presence of slightly negatively charged materials in the 0.2 M NaCl-Tris-HCl buffer (pH 8.0). Cytokines such as IL-1 α and IL-10 are produced by activated macrophages in mice,^{39,40} whereas TNF- α and IFN- γ are produced by activated macrophages and NK cells.^{40,41} IL-12, produced by macrophages, DCs, and APCs, activates NK cells.⁴² From the results of an immunoassay (Fig. 5) and fluorescence-activated cell sorting analyses (Fig. 6), we suggest that MLP-Fraction activated macrophages and DCs with the production of cytokines including IL- α , IL-10, IL-12, TNF- α , and IFN- γ . CD86 (B7-2) is expressed on APCs and acts as a ligand for CD28 on the surface of T-cells. B7-CD28 costimulation activates T-cells and induces a Th1-dominant response.⁴³ Although the level of CD69 expression on macrophages and DCs was increased by MLP-Fraction (Fig. 6A and B), CD86 expression levels were not significantly increased (data not shown). These results suggest that MLP-Fraction exhibited early activation of macrophages and DCs, compared with T-cell activation, after MLP-Fraction administration for 7 consecutive days. Therefore, we also investigated NK cells, and the results showed that treatment with MLP-Fraction increased the level of CD69 expression on NK cells (Fig. 6C and D). We determined the expression ratio of IFN- γ in NK cells to investigate which cells (NK cells or APCs, including macrophage and DCs) are the main producers of IFN- γ . MLP-Fraction treatment did not change the expression of IFN- γ in NK cells (data not shown) but did increase the expression of IFN- γ and IL-12 in APCs depleted of B-cells in normal mice (Fig. 7). We suggest that

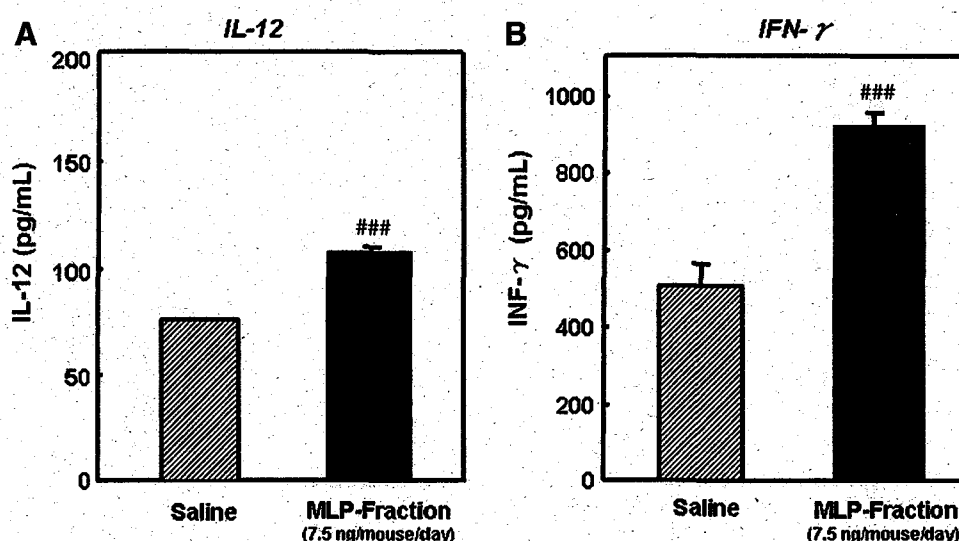


FIG. 9. Effect of MLP-Fraction on production of (A) IL-12 and (B) IFN- γ by splenocytes from colon-26 carcinoma-bearing mice. MLP-Fraction was administered (intraperitoneally) to two separate groups consisting of six colon-26 carcinoma-bearing BALB/c mice (6 weeks old) for 7 consecutive days. Splenocytes were collected on day 8, and the effects of MLP-Fraction on the production of IL-12 and IFN- γ by splenocytes were determined after a 24-hour incubation. Data are mean \pm SD values of two or three independent experiments ($n = 3$ per experiment). Significance of differences was tested using Student's t test. ### $P < .001$, in comparison with the saline-treated group.

IFN- γ is derived from activated macrophages and DCs in addition to IL-12. IL-12 is a Th1 cytokine that induces differentiation into Th1 cells and activates NK cells.⁴⁴ Figure 9 indicates that MLP-Fraction (administered over a 1-week period) would also induce a Th1-dominant response by which cytotoxic T-cells are activated for their cytotoxicity against cancer. As expected, MLP-Fraction showed an antitumor effect in tumor-bearing animals on day 8, and its TIR (67%) was higher than that of animals treated with polysaccharides such as MD-Fraction, lentinan, and PSK. The production of IL-12 and IFN- γ suggested that MLP-Fraction-activated NK cells through macrophages and DCs produced cytokines and simultaneously induced a Th1 response related to cytotoxic T-cells, which together contributed to reducing tumor size. A small amount of MLP-Fraction was enough to enhance immune responses in mice. This is the first study to show that a small protein or peptide from the maitake mushroom can act as an anticancer or anticancer-stimulating drug. Although we have not examined the administration of a combination of MLP-Fraction and MMC, we suggest that MLP-Fraction may restore the number of damaged immune cells in MMC-treated mice. This suggestion indicates the possibility that MLP-Fraction could reduce the necessary dosage of MMC, resulting in some relief from the side effects of MMC. In the present study, we demonstrated that our original protocol is useful for the discovery and screening of novel natural substances, with a focus on agents having small molecular weights, from mixtures of natural products isolated from edible mushrooms and medicinal plants. In future experiments, we need to completely purify and determine the structure of MLP-Fraction as well as the mechanism of its antitumor activity at molecular and cellular levels.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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