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NOTE

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Stimulation of the natural immune system in normal mice by polysaccharide from maitake mushroom

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Abstract Maitake D-Fraction is a polysaccharide extracted from the maitake mushroom (*Grifola frondosa* S.F. Gray). It is a β-glucan with a β-1,6 main chain with β-1,3 branches. Using normal C3H/Hej mice, its effects on the natural immune system, including macrophages, dendritic cells, and natural killer (NK) cells, were investigated. NK cells attack cells infected with pathogens such as bacteria and virus and produce cytokines, such as interferon-gamma (IFN-γ), that can modulate natural and specific immune responses. D-Fraction was administered to the mice intraperitoneally for 3 consecutive days; spleen cells containing macrophages and dendritic cells were then cultured and the culture supernatants were analyzed for IL-12. At the same time, IFN-γ expression in splenic NK cells was investigated. The levels of these cytokines were increased by D-Fraction. To elucidate NK cell activation by D-Fraction, CD69 expression on the surface of activated NK cells was examined, resulting in an increase in CD69-positive ratio for splenic NK cells. These results indicate that D-Fraction stimulates the natural immunity related to the activation of NK cells indirectly through IL-12 produced by macrophages and dendritic cells. Therefore, administration of D-Fraction to healthy individuals may serve to prevent infection.

Key words Maitake D-Fraction · Maitake mushroom · Natural immune system · NK cells

Introduction

Mushrooms have long been used as food and medical substances. The shiitake mushroom (*Lentinula edodes*, (Berk.) Sing), *Sclerotinia sclerotiorum* FKL, and *Schizophyllum commne* Fries have been examined for antitumor activity.

Several polysaccharides extracted from these mushrooms have been established as potent antitumor compounds. Lentinan from *Lentinula edodes* (Chihara et al. 1970), SSG from *Sclerotinia sclerotiorum* FKL (Ohno et al. 1987), and Schizophyllan from *Schizophyllum commne* Fries (Mitani et al. 1982) were found to enhance the immune response mediated by macrophages and T cells. In 1987, we detected antitumor activity in the edible maitake mushroom (*Grifola frondosa* S.F. Gray), which belongs to the Basidiomycetes, identified the responsible polysaccharide, and named it D-Fraction (Nanba et al. 1987). Many mushrooms contain various polysaccharides that inhibit tumor growth, most of which consist of β-1,3-glucan as a main chain with β-1,6 branches. In contrast, D-Fraction consists of a β-1,6-glucan as a main chain with β-1,3 branches. It showed good antitumor activity and specificity. Also, D-Fraction had an antitumor effect by oral administration on mice without side effects (Hishida et al. 1988). It has been commercialized since its safety was proven by Consumer Product Testing (NJ, USA). These events led us to a notion of using D-Fraction in healthy individuals to prevent infection by microorganisms.

The immune response is a mechanism that destroys and eliminates antigens from genetically nonidentical organisms as well as other foreign bodies, and its specific immunity is mainly involved in lymphocytes. The most common types of lymphocytes are B and T cells, of which T cells are restricted by the major histocompatibility complex (MHC). There are, however, unsensitized lymphocytes, found by Herberman et al. (1975) and designated natural killer (NK) cells, that are not restricted by the MHC, but can target and damage a variety of cells. NK cells form a unique third group of lymphocytes that differ from T and B cells in surface phenotype, target cell recognition, and function. NK cells have two relevant functions, related to the natural immune response against pathogens (Sepulveda and Puente 2000). One is cytotoxicity, mediated by the recognition and lysis of target cells such as virus and bacteria-infected cells. The second NK cell function is to produce cytokines, mainly

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IFN-γ, that can modulate natural and specific immune responses.

Cytotoxicity and cytokine secretion contribute to host resistance against microorganisms, and both functions are significantly altered in infectious diseases. Also, the immunity can be broadly categorized into acquired immunity and natural immunity. Acquired immunity is mediated by clonally distributed T and B cells and is characterized by specificity and memory. In contrast, natural immunity is a nonspecific immune response characterized by engulfment and digestion of microorganisms and foreign substances by macrophages and activation of NK cells. In preventive medicine, therefore, defense against invasion by foreign bodies is dependent on enhancing the natural immune system, including activating macrophages and NK cells. With the aim of eventually preventing infection or the proliferation of tumor cells by enhancing the natural immune system with D-Fraction, we investigated the activation of NK cells and its mechanism in normal mice administered D-Fraction.

Materials and methods

Male C3H/Hej mice (7-weeks-old) provided by Crea Japan (Tokyo, Japan) were raised for 1 week before being used for experiments. Food (CE-2; Crea Japan) and water were given freely to these animals until the experiments began.

The antibodies, purchased from PharMingen (San Diego, CA, USA) included, anti-CD16/CD32 2.4G2 monoclonal antibody (0.5 mg/ml), fluorescein isothiocyanate (FITC)-conjugated anti-CD69 H1.2F3 monoclonal antibody (0.5 mg/ml), phycoerythrin (PE)-conjugated anti-PanNK DX5 monoclonal antibody (0.2 mg/ml), Cy-Chrome-conjugated anti-CD3ε 145–2C11 monoclonal antibody (0.2 mg/ml), and FITC-conjugated anti-IFN-γ XMG1.2 monoclonal antibody (0.5 mg/ml).

Dried powder made from maitake mushrooms was obtained from Yukiguni Maitake (Niigata, Japan). The maitake D-Fraction (D-Fraction) was obtained from the powder by a method described previously (Shigesue et al. 2000). Briefly, the powder was heated with distilled water at 121°C for 60 min by autoclave. EtOH was added to the hot-water extract at a final concentration of 50% (v/v). After removal of the floating substances, the precipitate was recovered by centrifugation and separated by ion-exchange chromatography and gel filtration chromatography. Finally, a high molecular weight polysaccharide was obtained and named D-Fraction. The saccharide concentration was determined by the anthrone method (Dreywood 1946).

The D-Fraction (4.0 or 8.7 mg/kg/day) was administered to normal C3H/Hej mice (7 weeks old) intraperitoneally (i.p.) either only once or once a day for 3, 5, or 7 consecutive days. As a control, phosphate-buffered saline (PBS) was injected. Whole spleen cells were prepared from mice administered D-Fraction or PBS. Briefly the spleen was extirpated, passed through nylon mesh (ϕ 70 μm), and washed with Eagle's minimum essential medium (MEM) (Nissui Seiyaku, Tokyo, Japan). After centrifugation (300 *g*, 5 min, 4°C), the precipitated cells were collected and hemolyzed with hemolytic buffer (0.75% NH_4Cl in 0.1 N Tris-HCl buffer, pH 7.65) to remove erythrocytes. After further centrifugation, the precipitated cells were washed with RPMI-1640 medium (Nissui Seiyaku) and suspended in the same medium containing 10% fetal bovine serum (FBS) (Dainippon Seiyaku, Tokyo, Japan).

NK and T cells were stained for specific cell-surface markers on T cells ($CD3\varepsilon^+$) and NK cells ($PanNK^+$), respectively, then NK cells ($CD3\varepsilon^-PanNK^+$) were double stained for CD69 as the earliest activation marker on NK cells (Jovic et al. 2001; Gridley et al. 2002). For cell-surface antigen detection, 100 μl whole spleen cells (1×10^7/ml) was mixed with 1 μl anti-CD16/CD32 monoclonal antibody in a tube to block the Fc receptor and reacted at 4°C for 5 min. The mixture was incubated with FITC-conjugated mouse CD69 (0.5 mg/ml) monoclonal antibody, phosphatidylethanolamine (PE)-conjugated mouse PanNK monoclonal antibody or Cy-Chrome-conjugated CD3ε monoclonal antibody (mouse antibodies ≧1 μg) at 4°C for 35 min, washed with Washing Solution (0.09% NaN_3 and 1% FBS in PBS), then suspended in 0.5 ml Washing Solution and enumerated with a FACScan flow cytometer (Beckton Dickinson, Grenoble, France).

For intracellular cytokines, 2-ml aliquots of whole spleen cells (2×10^6/ml) were applied to 24-well plates, ionomycin (1 μg/ml) and phorbol-12-myristate-13-acetate (25 ng/ml) were added to each well, and the cells were then incubated with 2.8 μl Goldi Stop (PharMingen, San Diego, CA, USA) at 37°C in a 5% CO_2 atmosphere for 4 h. After the incubation, 0.5 μl CD16/CD32 monoclonal antibody to block the Fc receptor on the cell surface was mixed with the cells and reacted at 4°C for 5 min. Then, 2 μl each Cy-Chrome-conjugated CD3ε monoclonal antibody and PE-conjugated PanNK monoclonal antibody was added at 4°C for 20 min. After the reaction, the cells were washed with staining medium (SM: 0.05% NaN_3 and 1% FBS in PBS), incubated with 100 μl Cytofix/Cytoperm (PharMingen) at 4°C for 20 min, and washed again with Perm/Wash (PharMingen). The stained cells were reacted with 2 μl FITC-conjugated anti-IFN-γ antibody at 4°C for 30 min, washed with Perm/Wash, suspended in 50 μl SM, and enumerated using FACScan (Beckton Dickinson).

Whole spleen cells (2×10^5) were prepared from mice administered D-Fraction for 3 consecutive days and were cultured in a 96-well plate with concanavalin A (Con A; final concentration, 10 μg/ml) at 37°C for 36 h in 5% CO_2. After the Con A stimulation, culture supernatant (100 μl) was collected by centrifugation (300 *g*, 5 min), and the level of total IL-12 was determined with a mouse total IL-12 enzyme-linked immunosorbent assay (ELISA) kit (Genzyme, Minneapolis, MN, USA).

The data were examined for significance by the Mann–Whitney *U*-test. A *P* value of less than 0.05 was considered statistically significant.

Results and discussion

In our previous study, when the D-Fraction was administered to MM-46 carcinoma-bearing mice, the cytokine productivity by macrophages and T cells was enhanced and strong anti-tumor action was observed (Adachi et al. 1987; Inoue et al. 2002). Also, we found that when NK cells prepared from MM-46 carcinoma-bearing mice administered the D-Fraction were cultured, their relative cytotoxicity increased compared with the control mice (Adachi et al. 1987). To extend the application to preventive medicine, it is necessary to know whether D-Fraction enhances the activities of immunocompetent cells including macrophages, dendritic cells, and NK cells in the natural immune system of normal mice. Inactivated NK cells do not produce cytokines, but activated cells are a major producer of IFN-γ. Therefore, the level of intracellular IFN-γ in NK cells was investigated by flow cytometric analysis. The $CD3\varepsilon^-$ $PanNK^+$ cells that expressed PanNK but not CD3ε were assumed to be NK cells in the analysis because CD3ε and PanNK are antigens expressed on the surface of T cells and NK cells, respectively. After 5 consecutive days of administration, $PanNK^+$ IFN-γ^+ cells in whole spleen increased from 3.09% to 9.45%, as shown in a dot plot for PanNK and IFN-γ (Fig. 1A). During 7 days of administration, there was a significant difference between the control and D-Fraction-administered group at IFN-γ expression of NK cells in whole spleen (Fig. 1B). Next, NK cells prepared from whole spleen were cultured, the IFN-γ concentration in the culture supernatant was measured, and the increase in IFN-γ production caused by administration of D-Fraction was evaluated. The highest value was three times the control value on 3 consecutive days (data not shown). Administration of D-Fraction to normal mice increased IFN-γ positivity in the NK cells and secreted IFN-γ in the culture supernatant.

We considered that D-Fraction enhances the activation of NK cells either directly or through some intermediary reaction. To elucidate the mechanism of NK activation by D-Fraction, the degree of cellular activity was analyzed by detection of CD69 expression, as CD69 is an early-stage antigen expressed in NK cells. PBS or the D-Fraction at 8.7 mg/kg/day was administered i.p. to the C3H/Hej mice only once, and CD69 expression in splenic NK cells was examined by flow cytometric analysis. The percent positivity for CD69 in the NK cells increased to 1.4 times the control level in 18 h after the administration of D-Fraction (Fig. 2). A single administration resulted in an enhancement of CD69 expression in NK cells starting from 18 h compared with the PBS administration, and at 48 h, CD69 expression achieved a maximum. There is a significant difference between the control and D-Fraction-administration group from 20 to 72 h.

D-Fraction activates the immunocompetent cells including macrophages and T cells (Adachi et al. 1987; Hishida et al. 1988). Therefore, it seems that NK cells are activated through a metabolic agent or through assistance by cytokines produced seemingly by macrophages or other

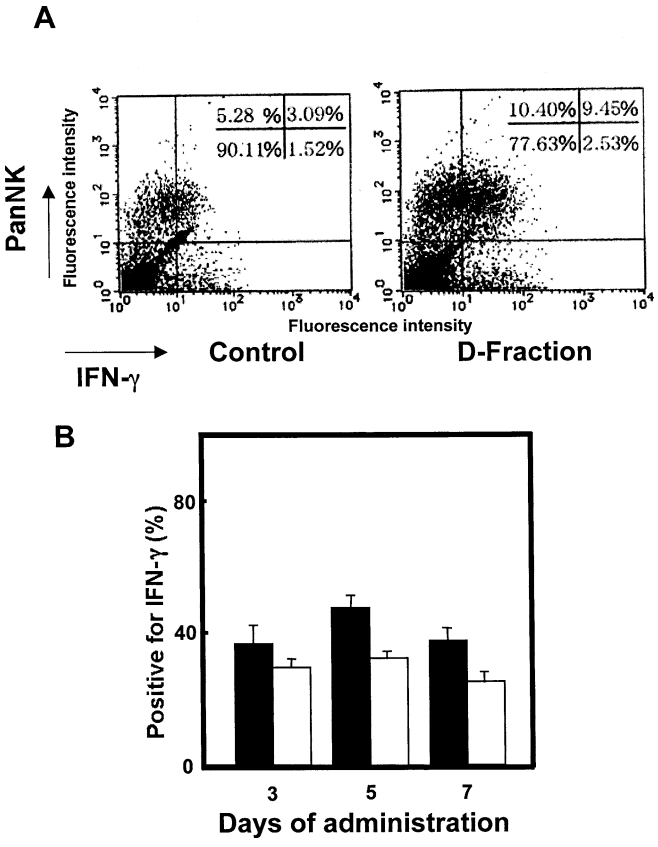

Fig. 1. Effects of D-Fraction on interferon (IFN)-γ expression in splenic natural killer (NK) cells of C3H/Hej mice. D-Fraction (8.7 mg/kg/day) or phosphate-buffered saline (PBS) was administered to the mice i.p. for 3, 5, or 7 consecutive days. Whole spleen was prepared, and IFN-γ expression in splenic NK cells ($CD3\varepsilon^-PanNK^+$cells contained in lymphocyte gate) was detected by flow cytometry. **A** Dot plot for Pan NK and IFN-γ expression after 5 consecutive days of administration. **B** Percent positivity for IFN-γ in splenic NK cells. *Open bars*, control; *Solid bars*, D-Fraction. All data are given as the mean ± SE of four experiments (3–4 mice)

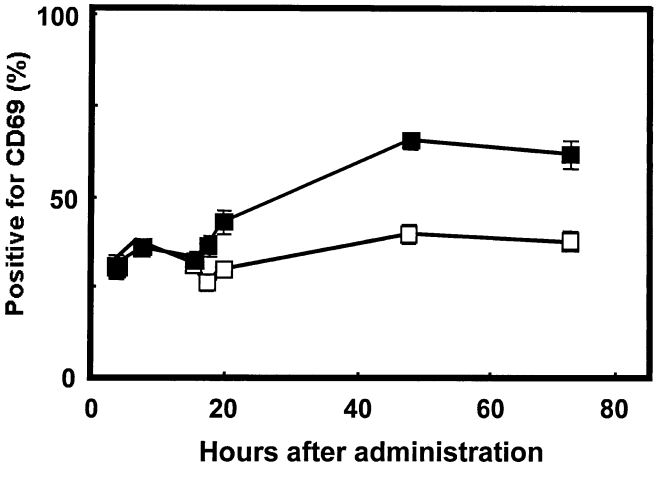

Fig. 2. Effect of a single administration of D-Fraction on CD69 expression on the surface in splenic NK cells of C3H/Hej mice. D-Fraction (8.7 mg/kg/day) or PBS was administered only once to the mice. After 72 h, whole spleen was prepared, and CD69 positivity in splenic NK cells ($CD3\varepsilon^-PanNK^+$cells contained in lymphocyte gate) was detected by flow cytometry and analyzed as percent positive. *Open squares*, control, *Closed squares*, D-Fraction. All data are given as the mean ± SE of four experiments (3–4 mice)

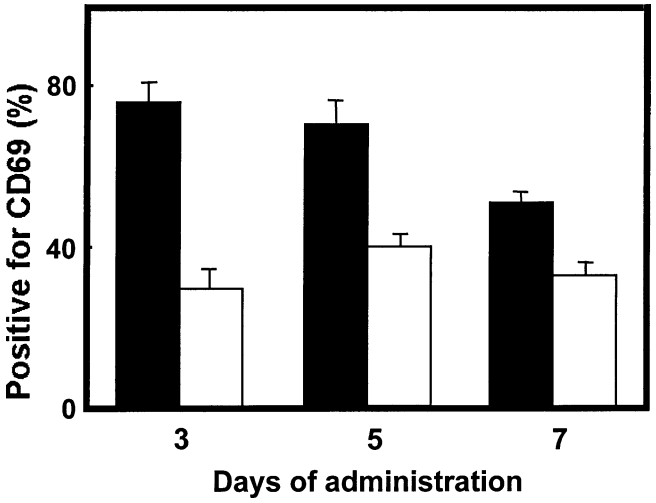

Fig. 3. Effect of consecutive administration of D-Fraction on CD69 expression on the surface of splenic NK cells of C3H/Hej mice. D-Fraction (8.7 mg/kg/day) or PBS was administered to the normal mice once a day for 7 days. Whole spleen was prepared, and CD69 positivity in splenic NK cells ($CD3\varepsilon^-PanNK^+$cells contained in lymphocyte gate) was detected by flow cytometry and analyzed as percent positive. *Open bars*, control; *solid bars*, D-Fraction. All data are given as the mean ± SE of four experiments (3–4 mice)

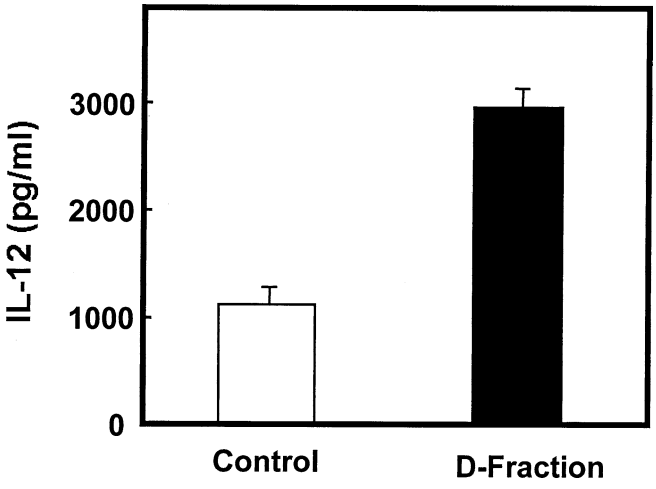

Fig. 4. Effect of D-Fraction on IL-12 production by whole spleen cells of C3H/Hej mice. D-Fraction (4.0 mg/kg/day) or saline was administered to normal mice once a day for 3 consecutive days. After the stimulation, the level of IL-12 in the culture supernatant was determined by ELISA. All data are given as the mean ± SE of three experiments (2 mice)

cells rather than by direct influence of the D-Fraction. To investigate the effect of a consecutive administration of D-Fraction on CD69 expression in splenic NK cells, we also administered D-Fraction once a day for 3, 5, or 7 consecutive days (Fig. 3). During 7 days of administration, the percent positivity for CD69 in the NK cells increased significantly compared with the control. The highest value was 2.6 times the control at day 3. The ratio showed 1.7 times increase as compared with the control at 48 h after a single administration of D-Fraction. The result indicates that a consecutive administration of D-Fraction has more effect for the activation of NK cells than does the single adminstration.

NK cells are activated by IL-12, which is produced by activated macrophages and dendritic cells (Lehman et al. 2001). CD69 expression in splenic NK cells experiments for both a single and a consecutive administration of D-Fraction increased compared with the control (Fig. 3). Therefore, to assay the activation of NK cells indirectly including macrophages and dendritic cells, D-Fraction was administered to normal mice for 3 consecutive days, and the level of IL-12 produced by whole spleen cells was measured by ELISA. During 3 days of administration, the level of IL-12 increased significantly compared with the control (Fig. 4). This result suggests that NK cells were activated through IL-12 produced by macrophages and dendritic cells activated by D-Fraction indirectly.

In conclusion, D-Fraction enhances the natural immune system including NK cells, macrophages, and dendritic cells in normal mice. Its application may help the host defense system against pathogens such as bacteria and viruses, reducing the possibility of human infection and disease. D-Fraction may thus be potentially useful in preventive medicine. In addition, tumor cell growth may be repressed by the cytotoxicity of NK cells activated by D-Fraction.

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