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A Polysaccharide, Extract From *Grifola frondosa*, Induces Th-1 Dominant Responses in Carcinoma-Bearing BALB/c Mice

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ABSTRACT—A polysaccharide, designated as the D-fraction, extracted from *maitake* (*Grifola frondosa*), was reported to have anti-tumor effects by activating macrophages and T cells in C3H/HeN mice in which a Th-1 dominant response was established. In this study using BALB/c mice in which a Th-2 response is genetically dominant, D-fraction reduced the expression of Th-2 cytokine interleukin (IL)-4 but markedly increased the expression of Th-1 cytokine interferon (IFN)-γ in $CD4^+$ T cells and also increased IL-12p70 production as well as IFN-γ production by antigen-presenting cells (APCs), suggesting that D-fraction promotes the differentiation into Th-1 cells of $CD4^+$ T cells through enhancement of IL-12p70 production by APCs.

Keywords: Polysaccharide, Th-1 dominance, BALB/c mice

In 1986, Mosmann classified $CD4^+$ T cell clones into helper (Th)-1 cells, which produce interleukin (IL)-2, interferon (IFN)-γ and tumor necrosis factor (TNF)-β and introduce cellular immunity to the organism, and Th-2 cells, which produce IL-4, IL-5, IL-6, IL-10 and IL-13 and activate humoral immunity (1, 2). A recent study showed that many immune responses are controlled by the proportion of Th-1 to Th-2 cells in humans as well as animals (3). Disruption of the balance between Th-1 and Th-2 induces an excessive Th-1 or Th-2 dominant response and causes various diseases (4). An excessive Th-1 response for intracellular infection by pathogens such as *Listeria* and *Spirochaeta* causes listeriosis and Lyme disease, and a Th-1 cell response activated by pancreaticβ-cell antigen or myelin basic protein activates macrophages excessively, which results in autoimmune diseases such as diabetes (type I) and multiple sclerosis, respectively, while a continuous Th-2 response induces allergic disease, asthma, and acquired immunodeficiency syndrome (AIDS). Therefore, it seems to be important to modulate the balance between Th-1 and Th-2 for treating disease.

We have already reported that D-fraction, a polysaccharide extracted from the fruiting bodies of the *maitake* mushroom (*Grifola frondosa*), activates cellular immunity and expresses anti-tumor effects (5 – 7). In addition, administration to MM-46 carcinoma-bearing C3H/HeN mice induced a Th-1 dominant response when D-fraction expressed its anti-tumor effects (8). However, there has been no investigation in BALB/c mice, in which a Th-2 response is genetically dominant. The BALB/c mouse is known to be susceptible to the intracellular pathogen *Leishmania* genetically, with a Th-2 response induced. In contrast, C57BL/6 and C3H/HeN mice are resistant to the bacteria, exhibiting Th-1 responses (9). In this study, the anti-tumor effect of D-fraction in colon 26-carcinoma-bearing BALB/c mice was investigated through association with $CD4^+$ T cell activation and induction of differentiation to Th-1 cells or Th-2 cells.

A dried powder made from the *maitake* mushroom was obtained from Yukiguni Maitake Co., Ltd. (Niigata). The D-fraction was prepared from the powder according to a method described previously (10). The saccharide concentration was determined by the anthrone method (11). The level of LPS contained in D-fraction was determined by using an Endospecy ES-20S Set (Seikagaku Iindustry Co., Tokyo), and the ratio (%) of LPS in D-fraction was less than 0.000007%.

Colon 26 carcinoma cells (1×10^5) were implanted in female BALB/c mice (6-week-old) in the right axillary region. After 24 h, D-fraction (7.8 mg/kg per day) or saline was administered to the carcinoma-bearing mice intraperitoneally (i.p.) for 19 consecutive days. On day 20, the tumor was extirpated and the weight was measured to

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obtain the tumor inhibition rate (T.I.R.) On day 20 in mice administered D-fraction or saline, the spleen and the inguinal lymph node were extirpated, and then spleen cells and lymph node cells were prepared by a method described previously (8). The antigen-presenting cells (APCs) were prepared from whole spleen cells or lymph node cells by using MACS Separation Columns with MHC class II (Ia) MicroBeads (Miltenyi Biotec. Co., Auburn, CA, USA).

For cytokine production, 1×10^6 cells/well of whole spleen cells or lymph node cells were cultured in RPMI-1640 medium containing 5% FBS with Con A (10 μg/ml) at 37°C for 24 h in 5% CO_2. After the stimulation, levels of IFN-γ and IL-4 in the culture supernatant were determined using mouse IFN-γ and IL-4 ELISA kits (Genzyme Co., Minneapolis, MI, USA). For APCs prepared from whole spleen cells or lymph node cells, a IL-12p70 ELISA kit (Genzyme Co.) was used.

The effect of D-fraction on colon 26 carcinoma cell growth was investigated for 20 days after tumor challenge as shown in Fig. 1A. There are significant differences between D-fraction-administered mice and control mice on days 12, 16 and 20. On day 20, tumors from D-fraction-administered mice weighed less than those from the control mice when the T.I.R. was 79%, indicating that D-fraction inhibited the cell growth (Fig. 1B). In a previous study using MM-46 carcinoma-bearing C3H/HeN mice, D-fraction inhibited carcinoma cell growth and showed a T.I.R. of 82%: D-fraction induced a Th-1 response and enhanced cellular immunity (8).

The current model of T cell activation requires two signals. The first signal is specific, requiring T cell receptor recognition and binding to MHC/antigen presented by APC. The second signal is nonspecific, resulting from the binding of B7 ligand on the APC with its receptor, CD28, on the T cell (12). However, it is unclear whether D-fraction enhances the CD28/B7 pathway. The preferential binding of the B7-CTLA4 (CD152) pathway results in the down-regulation of the responding T cells. The B7/CD28/CTLA4 pathway has the ability to both positively and negatively regulate immune responses (12). In this study, the effect of D-fraction on $CD4^+$ T cell activation was investigated for CD28 and CD152 expression. The ratio (%) of CD28 and CD152 expression on $CD4^+$ T cells from whole spleen and lymph node cell populations was analyzed by flow cytometry. As shown in Fig. 2, the ratio of CD28 expression on $CD4^+$ T cells increased significantly in both whole spleen cells and lymph node cells, while CD152 expression was unchanged. A previous paper on C3H/HeN mice reported the activation of $CD4^+$ T cells by D-fraction as determined by flow cytometric analysis for the expression of surface antigen CD69, which is known to be an early activation marker on $CD4^+$ T or $CD8^+$ T cells. Therefore, the activation of $CD8^+$ T cells was investigated, but neither CD28 nor CD152 expression was affected by D-fraction in whole spleen cells and lymph node cells (data not shown). These results suggest that D-fraction stimulates the differentiation into Th-1 or Th-2 cells of $CD4^+$ T cells associated with APCs in tumor-bearing BALB/c mice.

To investigate the effect of D-fraction on the balance of Th-1/Th-2 in colon 26 carcinoma-bearing BALB/c mice in which a Th-2 response is dominant, the levels of INF-γ and IL-4 production by whole spleen cells and lymph node cells were determined by the ELISA method. As shown in Fig. 3A, levels of IFN-γ increased significantly in compared with the control value. In contrast, the production by IL-4 by whole spleen cells was decreased by D-fraction. Furthermore, the ratio (%) of IFN-γ and IL-4 expression in $CD4^+$ T cells from whole spleen and lymph node cell

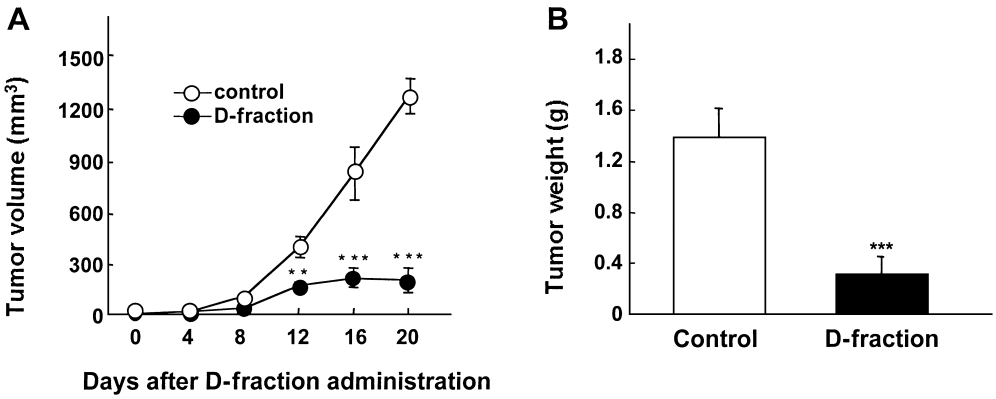

Fig. 1. Effects of D-Fraction on colon 26 carcinoma cell growth. D-Fraction (7.8 mg/kg per day) was administered to the colon 26 bearing-mice for 19 consecutive days, and tumor volume (A) and tumor weight (B) were determined. Values are expressed as the mean ± S.E.M. of 4 – 5 different experiments (5 – 7 mice/experiment). $^{**}P<0.01$, $^{***}P<0.001$, compared with the control group (Student *t*-test).

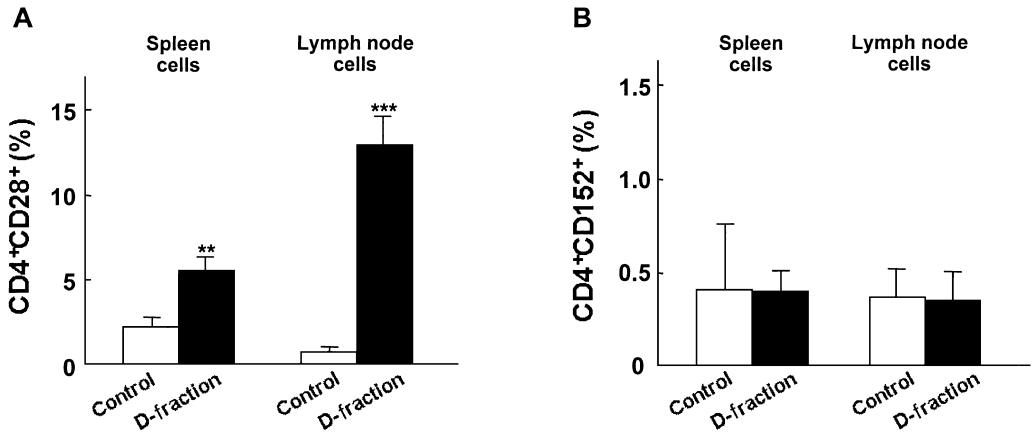

Fig. 2. Effects of D-Fraction on CD28 expression (A) and CD152 expression (B) on $CD4^+$ T cells. Values are expressed as the mean ± S.E.M. of 3 different experiments (2 mice/experiment). $^{**}P<0.01$, $^{***}P<0.001$, compared with the control group (Student *t*-test).

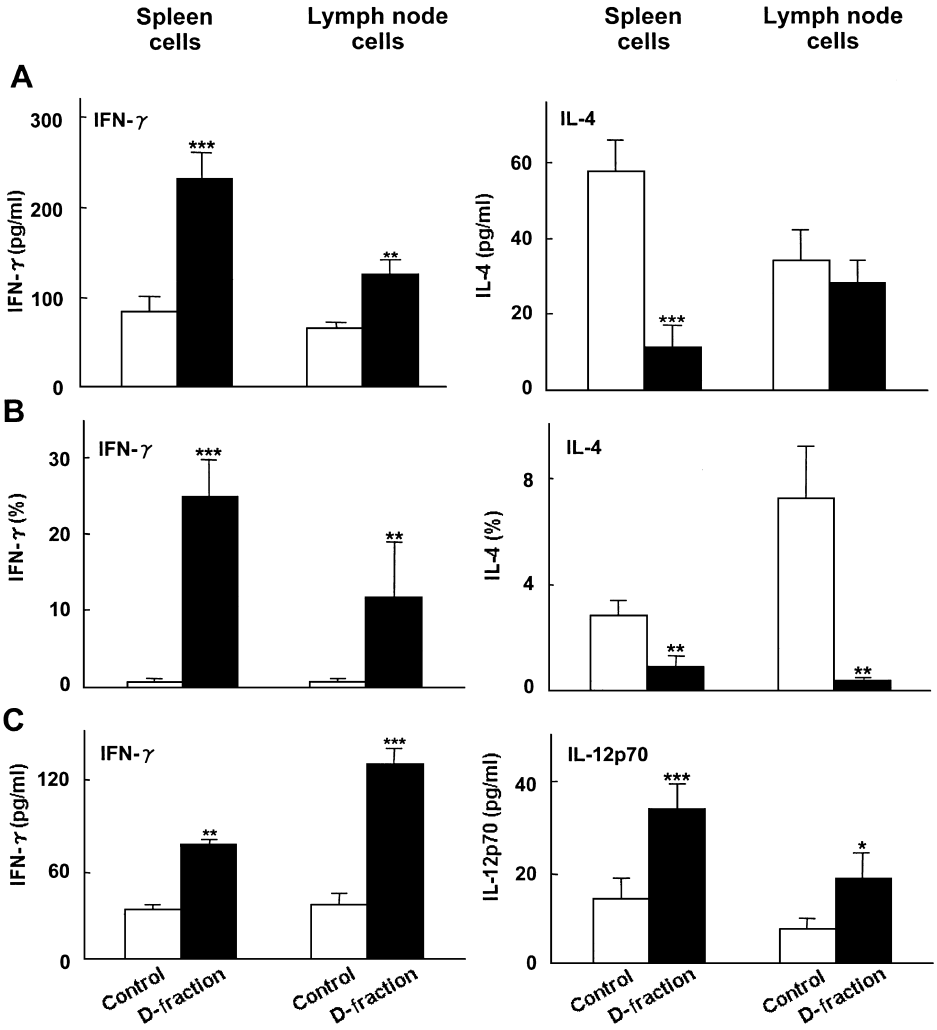

Fig. 3. Effects of D-Fraction on IFN-γ and IL-4 production (A), on intracellular IFN-γ and IL-4 expression in $CD4^+$ T cells (B) and on IFN-γ and IL-12p70 production by APCs (C). Values are expressed as the mean ± S.E.M. of different 3 – 4 experiments (3 – 4 mice/experiment). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared with the control group (Student *t*-test).

populations was analyzed by flow cytometry (Fig. 3B). The expression ratio for IFN-γ increased markedly compared with the control value, while that for IL-4 decreased. The ratio of IFN-γ/IL-4 in $CD4^+$ T cells was increased by D-fraction, indicating that D-fraction induced a Th-1 dominant response from strong Th-2 development due to carcinoma, which enhanced cellular immunity and expressed anti-tumor effects.

The differentiation of Th-1/Th-2 is known to involve cytokines such as IFN-γ (13) and IL-12p70 (14), produced by APCs. Notably, IL-12p70 induces differentiation into Th-1 cells, resulting in IFN-γ production, which enhances the Th-1 dominant response. The levels of IFN-γ and IL-12p70 production by APCs were determined by the ELISA method (Fig. 3C). The values for IFN-γ and IL-12p70 showed significant differences by treatment with D-fraction. These results suggest that D-fraction induces the differentiation into Th-1 cells of $CD4^+$ T cells in tumor-bearing BALB/c mice in which the Th-2 response was dominant through enhancement of IL-12p70 production by APCs such as macrophages and dendritic cells, which may support the possibility that D-fraction is a useful immunotherapeutic agent for patients with excessive Th-2 development such as allergic disease and asthma.

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