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Effect of Maitake (*Grifola frondosa*) D-Fraction on the Activation of NK Cells in Cancer Patients

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ABSTRACT Maitake D-Fraction, extracted from maitake mushroom, has been reported to exert its antitumor effect in tumor-bearing mice by enhancing the immune system through activation of macrophages, T cells, and natural killer (NK) cells. In a previous study, the combination of immunotherapy with the maitake D-Fraction and chemotherapy suggested that the D-Fraction may have the potential to decrease the size of lung, liver, and breast tumors in cancer patients. In the present study, we administered maitake D-Fraction to cancer patients without anticancer drugs, and at the same time NK cell activity was monitored to investigate whether the activity is closely related with disease progression. The numbers of $CD4^+$ and $CD8^+$ cells in the peripheral blood were measured in 10 patients, and NK cell activity was assessed using K-562 cells as target cells. Serum soluble interleukin-2 receptor (sIL-2R) levels in three patients and the expression of tumor markers in four patients were determined by enzyme-linked immunosorbent assay. The slight changes observed in the $CD4^+$ and $CD8^+$ cell numbers were independent of disease severity or stage as well as serum sIL-2R levels. In contrast, maitake D-Fraction hindered metastatic progress, lessened the expression of tumor markers, and increased NK cell activity in all patients examined. Thus maitake D-Fraction appears to repress cancer progression and primarily exerts its effect through stimulation of NK activity. In addition, we conclude that measurement of NK cell activity may be a useful clinical parameter in monitoring disease progression during and following immunotherapy with maitake D-Fraction.

KEY WORDS: • ***cancer patients*** • ***maitake D-Fraction*** • ***natural killer cells***

INTRODUCTION

THE FUNCTIONS of natural killer (NK) cell are broadly varied, from immunologic monitoring of tumors, providing defense against pathogens, and inducing proliferation of hemopoietic stem cells, to rejecting transplanted organs.[1] Pross and Maroun[2] standardized human NK cell assays for the sequential evaluation of patients with various disease states, or who are being treated with biological response modifiers. LeFever and Funahashi[3] reported that peripheral blood NK lytic activity in patients with bronchogenic carcinoma is significantly lower than in normal volunteers. Furthermore, when human recombinant β-interferon was administered daily or three times a week, NK activity was notably enforced and was maintained throughout the period of administration.[4] The effect of radiotherapy on peripheral blood NK cell numbers and activity has also been investigated.[5] These reports indicate that the decrease in NK cell activity observed in cancer patients is closely related to disease progression and declining immune function; thus changes in NK cell activity may be an effective clinical marker of disease progression in cancer patients.

We previously reported that the β-glucan termed D-Fraction, extracted from the fruit body of the maitake mushroom (*Grifola frondosa*), is a biological response modifier like lentinan, which is found in *Letinus edodes*.[6–8] In animal experiments, D-Fraction has been found to enhance the activity of immunocompetent cells such as macrophages, helper T cells, cytotoxic T cells, and NK cells. In addition, orally administered D-Fraction was shown to reduce tumor size in mice without causing unwanted side effects.[7] The safety of D-Fraction has been confirmed by the Consumer Product Testing Co. (Fairfield, NJ). Therefore, D-Fraction is widely sold as a nutritional supplement and touted as beneficial for health in Japan. We have already performed a nonrandomized clinical trial of D-Fraction treatment for patients with stage II–IV lung and breast cancer who gave informed consent.[9] In this trial, we found that interleukin-2 (IL-2) production is enhanced in D-Fraction-treated patients, suggesting that macrophages, T cells, and NK cells are activated by D-Fraction. D-Fraction has also been reported to be effective for HIV infection.[10] These results indicate that one mechanism by which D-Fraction enhances the antitumor immune response is stimulation of adaptive immunity, in particular, enhancement of cytotoxic T cell activation. In a re-

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cent clinical study, we found that D-Fraction enhances and maintains the peripheral blood NK cell activity of cancer patients after administration.[11] However, it is unknown whether D-Fraction predominately affects NK cell activity or cytotoxic T cells.

In the present study, D-Fraction was administered to six male and four female patients between the ages of 46 and 84 years, with stage II–IV lung, breast, lingual, or gastric cancer who were not undergoing conventional treatments such as chemotherapy or radiotherapy. The $CD4^+/CD8^+$ cell numbers, NK cell activity, levels of soluble IL-2 receptor (sIL-2R), and expression of tumor markers such as carcinoembryonic antigen (CEA), carbohydrate antigen 15-3 (CA15-3), and carbohydrate antigen 19-9 (CA19-9) were all monitored as measures of disease progression. The present results suggest treatment of severe or late-stage cancer patients with D-Fraction as a means of stimulating antitumor immunity.

MATERIALS AND METHODS

Materials

The dried powder made from the fruit body of maitake mushrooms was purchased from Yukiguni Maitake Co., Ltd. (Niigata, Japan). K-562 cells (the chronic myelogenous leukemia cell variety) were purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). For detection of human NK cell activity, chromium-51 (Daiichi Kagaku Yakuhin Co., Ltd., Tokyo, Japan) and Lymphosepar ($d = 1.077$) (Immuno Biological Lab., Co., Ltd., Fujioka, Japan) were prepared.

Preparation of D-Fraction

The hot-water-soluble fraction of dried maitake mushroom powder was obtained by heating at 100°C for 30–46 minutes. Ethanol was added to the hot-water-soluble fraction at a final concentration of 50%, and the solution was stored at 4°C for 12 hours. After removing floating material, precipitate was obtained by centrifugation at 7,000 *g* for 10 minutes. The precipitate was soluble in distilled water, and was administered to cancer patients as D-Fraction. Polysaccharide concentration was determined by the anthrone method.[12]

Administration of D-Fraction

Ten patients with stage II–IV cancer ranging in age from 46 to 84 years who agreed to the clinical trial as shown in Table 1 were given daily doses, twice a day, in morning and evening, of D-Fraction per day, 40 mg and 80 mg (for Patients A, C, E, and G), 50 mg and 100 mg (for Patients B and D), or 75 mg and 150 mg (for Patients F, H, I, and J), for from 1 to 63 months in duration. All patients gave informed consent to Dr. K. Komuta of Osaka Police Hospital.

Detection of human NK cell activity

NK cells (1×10^6) obtained from peripheral blood by the Conray-Ficoll method[13] were mixed with 2×10^7 ^{51}Cr-labeled K-562 target cells in a microplate. After incubation for 1 hour, ^{51}Cr release into the culture supernatant was detected with a γ-counter, and NK cell activity was calculated as follows: NK activity (%) = (experimental release − spontaneous release)/(maximum release with 1 *N* HCl treatment − spontaneous release) × 100.

Detection of sIL-2R

Levels of sIL-2R in peripheral blood of cancer patients were detected in serum samples with the DPC · immuriged IL-2R kit (Euro/DPC Ltd., Gwynedd, Wales, UK).

Detection of $CD4^+$ cells and $CD8^+$ cells

Phosphate-buffered saline (100 μL) was mixed with a peripheral blood sample (100 μL) in a tube, and fluorescein isothiocyanate-conjugated CD4 monoclonal antibody (DAKO Japan Co., Ltd., Kyoto, Japan) or fluorescein isothiocyanate-conjugated CD8 monoclonal antibody (Nichirei Co., Ltd., Tokyo) was added. The mixture was incubated for 15 minutes in the dark and was then hemolyzed to remove red blood cells. After the centrifugation at 800 *g* for 10 minutes, the cells were collected and resuspended in

Table 1. Cases of Advanced Cancer

Patient	*Age (years)*	*Sex*	*Cancer*[a]	*Stage*[a]	*Metastasis*[a]
A	62	M	Lung (small cell carcinoma)	IV	ND
B	59	F	Lingual[b]	III	ND
C	74	M	Lung (small cell carcinoma)	III	ND
D	54	M	Lung (squamous cell carcinoma)	IV	Lymph node
E	46	F	Breast (small cell carcinoma)	IV	Lung
F	52	M	Gastric (small cell carcinoma)	IV	Lung
G	47	F	Lung (small cell carcinoma)	II	ND
H	69	M	Liver (tubular adenocarcinoma)	III	Lymph node
I	84	F	Breast (solid tubular carcinoma)	II	ND
J	53	M	Lung (small cell carcinoma)	III	ND

[a]Diagnosis prior to D-Fraction administration.
[b]After the removal of lingua.

Table 2. Effect of D-Fraction on $CD4^+$ Cell Percentage in Peripheral Blood of Cancer Patients

	Percentage of $CD4^+$ cells (date)[a]	
Patient	*Before treatment*	*After treatment*
A	43 ('99.8)	47 ('99.10), 45 ('00.1), 49 ('00.7), 45 ('01.1)
B	50 ('98.8)	46 ('98.9), 58 ('99.4), 49 ('99.10), 40 ('01.7), 51 ('01.9), 46 ('02.1)
C	53 ('99.6)	51 ('99.9), 53 ('99.11), 54 ('00.8), 57 ('00.12), 55 ('01.7), 48 ('01.9), 54 ('01.12)
D	22 ('00.4)	23 ('00.5), 24 ('00.12), 30 ('01.6), 27 ('01.9), 23 ('02.1), death ('02.2)
E	40 ('99.1)	38 ('99.6), 38 ('00.6), 38 ('01.7), 38 ('02.3)
F	24 ('97.11)	41 ('98.2), 21 ('98.5), 20 ('98.6), 19 ('98.8), 21 ('98.11), 20 ('99.1), 19 ('99.11), 18 ('99.12), 21 ('00.2), 22 ('00.5), 23 ('00.8), 21 ('01.7), 21 ('01.9), 20 ('01.11),
G	23 ('96.11)	36 ('96.12), 35 ('97.2), 21 ('98.5), 34 ('98.7), 35 ('98.10), 36 ('00.1), 35 ('01.2)
H	28 ('96.10)	35 ('97.4), 31 ('97.12), 32 ('98.4), 38 ('99.9), 30 ('01.3), death ('02.1)
I	29 ('97.9)	33 ('97.12), 34 ('98.4), 35 ('99.1)
J	40 ('98.4)	44 ('98.8), 43 ('99.7), 42 ('00.7), 47 ('00.9), 45 ('01.8)

[a]The percentage of total lymphocytes positive for CD4 was determined by flow cytometric analysis. Normal values range from 32.8% to 48.7%.

1 mL of phosphate-buffered saline. The cell suspension analyzed by flow cytometry (Ortho-clinical Diagnostics Co., Ltd., Tokyo), and the percentage of total lymphocytes positive for CD4 or CD8 was determined.

Detection of tumor markers

Levels of CEA, CA15-3, and CA19-9 in peripheral serum samples from cancer patients were detected with the SphereLight 180 kit (Olympus Optical Co. Ltd., Tokyo).

RESULTS

Effect of D-Fraction on $CD4^+$ and $CD8^+$ cell numbers in cancer patients

In a previous study, administration of D-Fraction to tumor-bearing mice activated $CD4^+$ and $CD8^+$ T cell subsets, resulting in enhanced cellular immunity.[14] Furthermore, the increase in the percentage of $CD4^+$ cells, which is higher than that of $CD8^+$ cells, indicates that D-Fraction markedly affects their activation. To obtain similar data on the effects of D-Fraction in human cancer patients, the percentages of $CD4^+$ and $CD8^+$ cells were estimated by flow cytometric analysis (Tables 2 and 3). The percentages of lymphocytes positive for CD4 and CD8 cells were increased slightly and maintained, although the levels were seen to decrease slightly throughout administration of D-Fraction. In all patients, $CD4^+$ and $CD8^+$ cell percentages were near or within the range of normal. However, Patients D and H died, suggesting that the monitoring of $CD4^+$ and $CD8^+$ cell percentages cannot accurately estimate cancer progression in patients during immunotherapy with D-Fraction.

Effect of D-Fraction on serum sIL-2R levels in cancer patients

The receptor for IL-2 consists of α, β, and γ subunits. It is commonly held that the sole role of the α subunit is in the generation of a high-affinity receptor complex.[15] The

Table 3. Effect of D-Fraction on $CD8^+$ Cell Percentage in Peripheral Blood of Cancer Patients

	Percentage of $CD8^+$ cells (date)[a]	
Patient	*Before treatment*	*After treatment*
A	30 ('99.8)	29 ('99.10), 31 ('00.1), 34 ('00.7), 35 ('01.1)
B	25 ('98.8)	27 ('98.9), 25 ('99.4), 32 ('99.10), 17 ('01.7), 23 ('01.9), 26 ('02.1)
C	33 ('99.6)	34 ('99.9), 31 ('99.11), 29 ('00.8), 31 ('00.12), 29 ('01.7), 27 ('01.9), 28 ('01.12)
D	25 ('00.4)	28 ('00.5), 26 ('00.12), 25 ('01.6), 28 ('01.9), 30 ('02.1), death ('02.2)
E	33 ('99.1)	38 ('99.6), 36 ('00.6), 35 ('01.7), 37 ('02.3)
F	31 ('97.11)	20 ('98.5), 19 ('98.6), 22 ('98.8), 24 ('98.11), 20 ('99.1), 30 ('99.11), 30 ('99.12), 31 ('00.2), 27 ('00.5), 33 ('00.8), 21 ('01.7), 30 ('01.9), 31 ('01.11)
G	39 ('96.11)	47 ('96.12), 33 ('97.2), 17 ('98.5), 23 ('98.7), 27 ('98.10), 38 ('00.1), 39 ('01.2)
H	51 ('96.10)	35 ('97.4), 35 ('97.12), 39 ('98.4), 33 ('99.9), 29 ('01.3), death ('02.1)
I	20 ('97.9)	30 ('97.12), 31 ('98.4), 29 ('99.1)
J	17 ('98.4)	20 ('98.8), 19 ('99.7), 19 ('00.7), 19 ('00.9), 19 ('01.8)

[a]The percentage of total lymphocytes positive for CD8 was determined by flow cytometric analysis. Normal values range from 18.9% to 32.6%.

subunit is released from the surface of macrophages, lymphocytes, and neutrophils, and can be measured as sIL-2R. The release of sIL-2R appears to characteristic of T lymphocyte activation and may serve an immunoregulatory function during both normal and abnormal cell growth and differentiation. Levels of sIL-2R were determined in Patients B, F, and G (Fig. 1). The levels of sIL-2R in Patients B and G were in the range normal range, 145–519 U/mL, and varied little over time. In contrast, the levels of sIL-2R in Patient F increased markedly after 7 months of D-Fraction administration. The sIL-2R values for Patient F increased above those of Patients B and G during D-Fraction administration, while the $CD4^+$ cell percentage for Patient F decreased to below normal (Table 2).

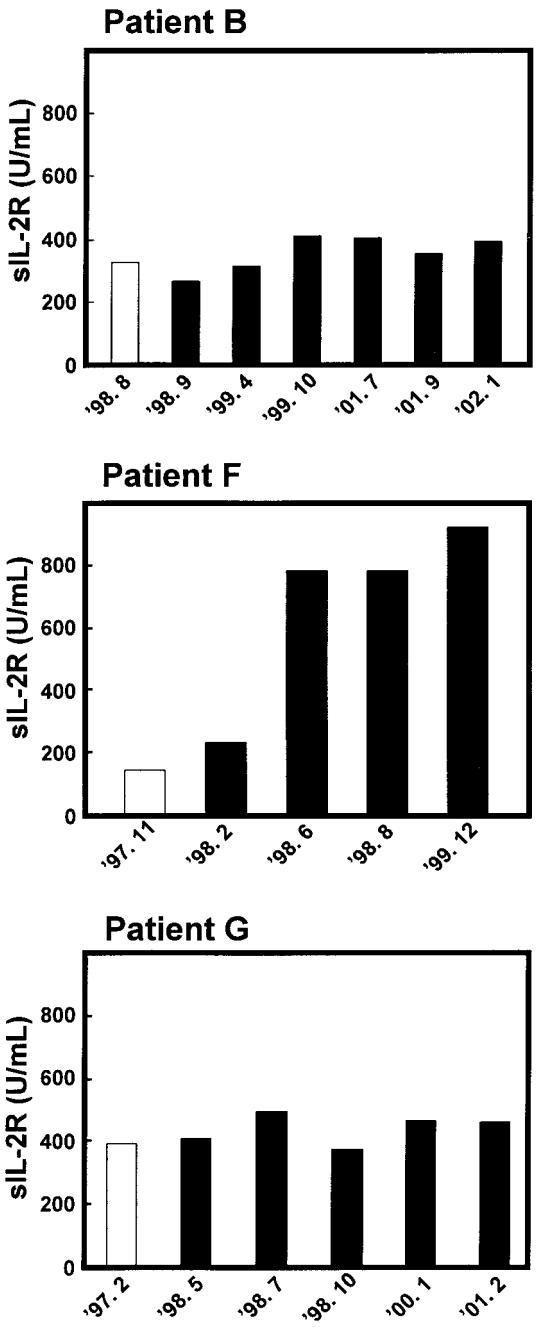

FIG. 1. Effect of D-Fraction on serum sIL-2R levels in cancer patients. After D-Fraction administration, levels of serum sIL-2R were determined by enzyme-linked immunosorbent assay. Normal values are 145–519 U/mL.

Effect of D-Fraction on the tumor marker in cancer patients

Patients A, F, H, and I were investigated for changes in tumor markers and NK cell activity as shown in Fig. 2. The CEA values for these four patients showed a decrease to within normal parameters (0–5 ng/mL) during D-Fraction administration. In Patient H, the level of CA19-9 decreased dramatically after D-Fraction administration and then was maintained at less than 37 U/mL during 3 years of administration. However, levels of both CEA and CA19-9 in Patient H were found to increase again and 18 months later when metastasis appeared in the brain, resulting in death in 10 months. In Patient F, the level of CA19-9 showed a gradual decrease corresponding to the increase of NK activity after D-Fraction administration, although the value of CA19-9 was not in the normal range. However, the decrease of NK cell activity was seen, related with the disease progression.

Effect of D-Fraction on NK cell activity in cancer patients

The decrease in peripheral blood NK cell activity in cancer patients is closely related to disease progression and declining immune function; thus changes in activity may be used as an effective clinical marker of disease progression.[1–5] Therefore we examined the enhancement of NK cell activity by D-Fraction in 24 cancer patients who gave informed consent. For 14 of the patients, D-Fraction was administered concomitantly with various food supplements, radiotherapy treatment, or chemotherapy. Therefore, NK cell activity was examined in the remaining 10 patients who were only given D-Fraction (Table 4 and Fig. 3). After administration of D-Fraction, dramatic increases in NK cell activity were seen. Although NK cell activity reached a maximum and then decreased slowly, activity was maintained at a higher level than observed prior to D-Fraction administration, except for Patients D and H, who later died. These results indicate that D-Fraction enhances and maintains NK cell activity in cancer patients. Although potential differences in the effects of D-Fraction between males and females were examined, none was observed.

DISCUSSION

Following most antitumor treatments, such as chemotherapy, radiation therapy, and surgical resection, the function of the immune system in cancer patients is often reduced. In contrast, immunotherapies such as IL-2 or β-interferon reverse the decline in T cell and NK cell activity.[16,17] D-

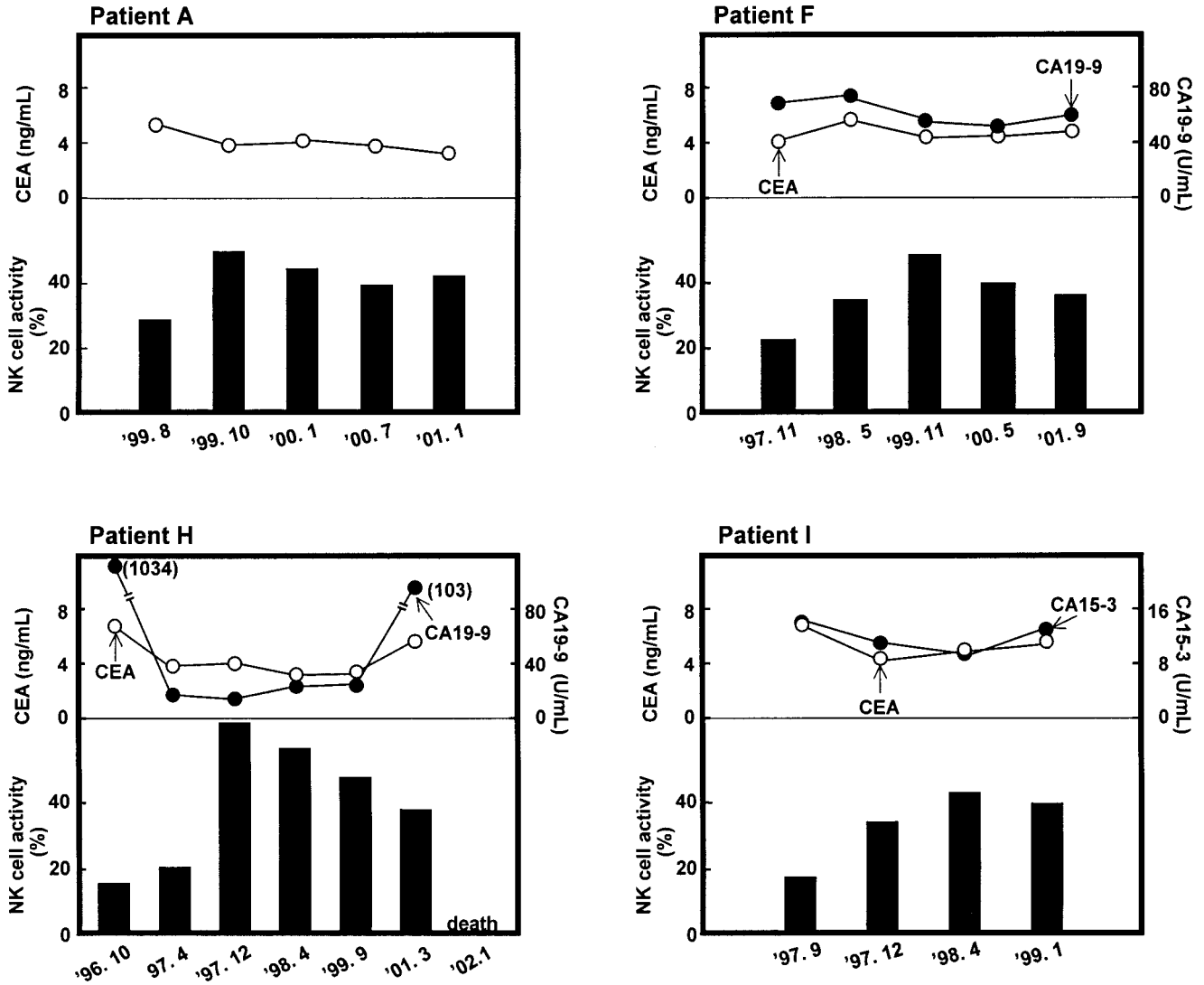

FIG. 2. Effect of D-Fraction on tumor marker expression and NK cell activity. Normal values are as follows: CEA, <5.0 ng/mL; CA19-9, <37 U/mL; CA15-3, <27 U/mL; NK cell activity, 18–40%.

TABLE 4. EFFECT OF D-FRACTION ON NK CELL ACTIVITY

	Percentage of NK cell activity (date)	
Patient	*Before treatment*	*After treatment*
A	29 ('99.8)	49 ('99.10), 45 ('00.1), 39 ('00.7), 42 ('01.1)
B	36 ('98.8)	48 ('98.9), 44 ('99.4), 39 ('99.10), 43 ('01.7), 37 ('01.9), 37 ('02.1)
C	23 ('99.6)	38 ('99.9), 46 ('99.11), 44 ('00.8), 62 ('00.12), 58 ('01.7), 41 ('01.9), 50 ('01.12)
D	30 ('00.4)	67 ('00.5), 51 ('00.12), 34 ('01.6), 27 ('01.9), 25 ('02.1), death ('02.2)
E	21 ('99.1)	28 ('99.6), 37 ('00.6), 39 ('01.7), 38 ('02.3)
F	22 ('97.11)	34 ('98.2), 27 ('98.5), 25 ('98.6), 20 ('98.8), 51 ('98.11), 46 ('99.1), 48 ('99.11), 33 ('99.12), 38 ('00.2), 40 ('00.5), 38 ('00.8), 39 ('01.7), 36 ('01.9), 39 ('01.11)
G	31 ('96.11)	27 ('96.12), 37 ('97.2), 48 ('98.5), 64 ('98.7), 60 ('98.10), 52 ('00.1), 48 ('01.2)
H	15 ('96.10)	20 ('97.4), 65 ('97.12), 57 ('98.4), 48 ('99.9), 38 ('01.3), death ('02.1)
I	17 ('97.9)	34 ('97.12), 43 ('98.4), 40 ('99.1)
J	16 ('98.4)	51 ('98.8), 42 ('99.7), 57 ('00.7), 47 ('00.9), 51 ('01.8)

Normal values range from 18% to 40%.

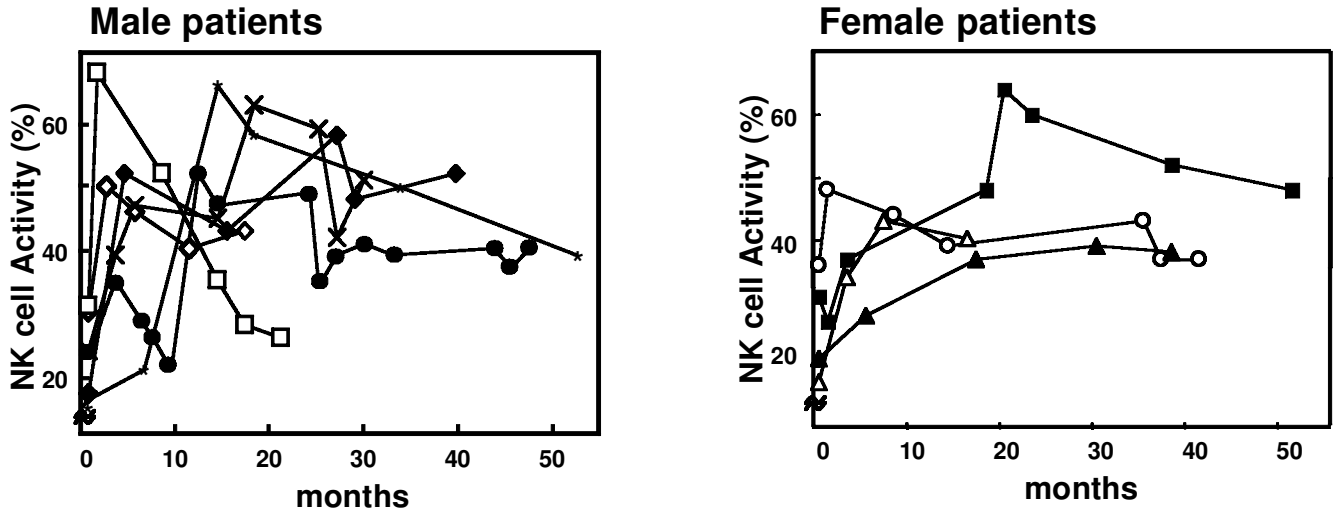

FIG. 3. Effect of D-Fraction on NK cell activity in cancer patients. After D-Fraction administration, activity of peripheral blood NK cells was determined by flow cytometric analysis in male patients A (◇), C (×), D (□), F (●), H (∗), and J (◆) and female patients B (○), E (▲), G (■), and I (△). Normal values are 18–40%.

Fraction, extracted from the fruit body of the maitake mushroom (*G. frondosa*), is a biological response modifier like lentinan from *L. edodes*.[6–8] In animal experiments, D-Fraction has been shown to enhance the activity of immunocompetent cells such as macrophages, helper T cells, cytotoxic T cells, and NK cells, resulting in reduced tumor size in mice, without causing unwanted side effects.[7] In this study D-Fraction therapy was administered to 10 patients with stage II–IV lung or breast cancer, and then $CD4^+$ and $CD8^+$ cell numbers, NK cell activity, and serum levels of sIL-2R were monitored. No new metastases were found in any patients given D-Fraction except for Patient H. As shown in Tables 2 and 3, D-Fraction was effective in slightly altering the $CD4^+$ and $CD8^+$ cell numbers. In contrast, NK cell activity was significantly elevated after D-Fraction administration and was maintained in the normal range (18–40%). The NK cell activity of Patients D and H decreased more markedly than those of other patients after reaching their maximal levels, and the declines correlated with the deaths of these patients.

Not only is sIL-2R used as a marker for human T cell leukemia virus I and non-Hodgkin's lymphoma, but also it appears to provide an additional serologic measure for assessing clinical progression in patients with HIV infection and AIDS.[18,19] Levels of sIL-2R have been reported to be increased in the blood of patients with autoimmune or inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, or Type 1 diabetes, and in infections such as hepatitis or HIV, as well as during transplantation rejection.[19] Patient F presented with gallbladder cancer (36 years old), and then developed adenoid cystic carcinoma. After removal of the whole maxilla (39 years old), pancreatic duodenectomy (42 years old), then removal of two-thirds of the stomach (45 years old), and appearance of metastasis to the lung (51 years old), D-Fraction therapy was started (52 years old). Patient F showed high-level sIL-2R even after D-Fraction administration (Fig. 1), suggesting disease progression, although T cell subset numbers, NK cell activity, and tumor markers were all within the normal range, and no further metastases were detected.

In conclusion, D-Fraction may inhibit cancer progression even without adjunct therapies. The results of the present study suggest that D-Fraction reverses the decrease in T cell number and activity seen in cancer, and is capable of enhancing and maintaining peripheral blood NK cell activity in patients with lung and breast cancer. Oral administration of D-Fraction is a pain-free treatment method for the patient and may be an effective method of stimulating the immune system to fight cancers, although further research on different types of cancers is needed.

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