Natural killer (NK) cells are directly cytotoxic for tumor cells and play a primary role in regulating immune responses. We monitored levels of NK cell cytotoxic activity in cancer patients receiving D-Fraction extracted from maitake mushrooms (Grifola frondosa). Elevated levels of cytotoxic activity were maintained for one year. To elucidate the mechanisms underlying long-term activation of NK cells during treatment with D-Fraction, we examined tumor volume and levels of IFN-\( \gamma \) and TNF-\( \alpha \) in MM46-bearing C3H/HeN mice to which D-Fraction was administered for 19 d. D-Fraction markedly suppressed tumor growth, corresponding with increases in TNF-\( \alpha \) and IFN-\( \gamma \) released from spleen cells and a significant increase in TNF-\( \alpha \) expressed in NK cells. This suggests that the D-Fraction activates NK cells even on the 20th day after treatment. Furthermore, D-Fraction increased macrophage-derived interleukin (IL)-12, which serves to activate NK cells. These results suggest that NK cells are not only responsible for the early effects of D-Fraction on tumor growth, but also for the long-term tumor-suppressive effects of D-Fraction through increased IL-12 released from macrophages.

Key words  IL-12 production; macrophage; NK cell

MATERIALS AND METHODS

**Materials** For the detection of human NK activity, chromium-51 (Daichi Kagaku Yakuhin Co., Tokyo) and lymphopacel (d=1.077) (IBL Co., U.S.A.) were prepared.

**Animals** Male C3H/HeN mice (4-weeks-old) were obtained (Japan Crea Co., Osaka) and were raised for one week before being used for experiments. Food and water were given freely to these mice until used for experiments.

**Cells** MM-46 carcinoma cells were kindly donated by Dr. Kanki Komiyama. \( {^{51}} \)Cr labeled K-562 cells were used as target cells of human NK.

**Preparation of D-Fraction** D-Fraction was prepared from the dried powder of the fruit body of maitake mushrooms (Grifola frondosa) (Yukiguni Maitake Co., Niigata), according to the method described in our previous paper.\(^6\) The level of LPS contained in D-Fraction was determined by using Endospecy ES-20S Set (Seikagaku Industry Co., U.S.A.) and the ratio (%) of LPS in D-Fraction was less than

**Fig. 1.** Chemical structure of D-Fraction
0.000007%. In the experiment using macrophage cell line RAW 264.7 cells, D-Fraction was pretreated with polymixin B (30 μg/ml) for 2 h.

**Administration of D-Fraction to Cancer Patients** Dr. Kiyoshi Komuta of the Osaka Police Hospital gave informed consent to 8 cancer patients. Eight stage II—IV cancer patients between 43- and 74-years-old were orally administered 100 mg of D-Fraction on consecutive days for 34 months and their NK activity was examined.

**Administration of D-Fraction to MM46 Carcinoma-Bearing C3H/HeN Mice** MM-46 carcinoma cells (2×10⁶) were implanted in the right axillary region of 5-week-old male C3H/HeN mice. After 24 h, D-Fraction (5 mg/kg/d) was administered to MM-46 carcinoma-bearing mice intraperitoneally (i.p.) for 19 consecutive days. Tumor inhibition ratio (T.I.R.) was calculated as follows: [1-(weight of tumor mass from mice treated with D-Fraction)/(weight of tumor mass from mice treated with PBS)]×100.

**Detection of Human NK Cell Activity** NK cells (1×10⁶) obtained from blood using the Conray–Ficoll method⁹ were mixed with 2×10⁷ of ⁵¹Cr-labelled K-562 (target cell of NK cell) cells in a tube. After incubation for 1 h, release of ⁵¹Cr into the culture supernatant was detected using a γ-counter. NK activity was calculated as: NK activity (%) = [(experimental release—spontaneous release) cpn/(max-release—spontaneous release) cpn (by 1N HCl treatment)]×100.

**RESULTS AND DISCUSSION**

**Effects of D-Fraction on MM46-Carcinoma Cell Growth** On the 20th day of D-Fraction administration to MM-46 tumor-bearing mice, D-Fraction significantly decreased tumor growth as compared with mice administered phosphate buffer saline (PBS), when the T.I.R. was 82% (Fig. 2). The effect of D-Fraction was also investigated in C3H/HeJ mice that are non-response to LPS; the T.I.R. was 78%. These results indicated that D-Fraction inhibited the growth of tumor.

**Effects of D-Fraction on NK Cell Activation** D-Fraction has already been reported to enhance cellular immunity by stimulating cells such as macrophages, helper T cells and cytotoxic T cells.⁵ To detect NK cell activation, we examined IFN-γ and TNF-α release from whole spleen cells on the 20th day of D-Fraction administration by mouse IFN-γ and TNF-α ELISA kits (Genzyme Co., Minneapolis, U.S.A.). As shown in Figs. 3A and 3B, levels of IFN-γ and TNF-α were significantly increased by 1.5- and 2.5-fold, respectively, compared to control mice. Intracellular expression of TNF-α in splenic NK cells under D-Fraction administration was also investigated using flow cytometric analysis. To detect NK cells, 2×10⁶ cells were stained with R-PE-conjugated PanNK and Cy-Chrom™-conjugated CD3ε antibodies (PharMingen Co., San Diego, CA, U.S.A.) and CD3ε negative and PanNK positive was determined to represent the NK cell. Fluorescein isothiocyanate (FITC)-conjugated TNF-α antibody (PharMingen Co.) was used for detecting TNF-α expression in NK cells. After staining, NK cells expressing TNF-α were analyzed using a FACSCalibur analyzer (Becton Dickinson Co., Grenoble, France) and histograms were calculated with FlowJo software (Becton Dickinson, Mountain View, CA, U.S.A.). As shown in Fig. 3C, D-Fraction administration resulted in a 1.3-fold increase in intracellular TNF-α expression in splenic NK cells compared to control mice. TNF-α is a cytokine released from activated NK cells in the same way as IFN-γ,¹⁰,¹¹ and has various functions relating to inflammatory and cytotoxic reactions, in addition to cytotoxicity and the ability to directly cause hemor-
Table 1. Activities of NK Cells in Cancer Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Before(b) (date)</th>
<th>After(c) (date)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCLC (small cell lung carcinoma) (74-year-old female, stage III)</td>
<td>23% (*99.6)</td>
<td>44% (*99.9), 51% (*00.2)</td>
</tr>
<tr>
<td>SCLC (small cell lung carcinoma) (54-year-old male, stage III)</td>
<td>21% (*98.5)</td>
<td>42% (*99.7), 47% (*00.9)</td>
</tr>
<tr>
<td>Alveolar cell carcinoma (54-year-old female, stage III-B)</td>
<td>24% (*98.4)</td>
<td>48% (*98.9), 44% (*99.4), 42% (*00.5), 44% (*01.2)</td>
</tr>
<tr>
<td>Pulmonary adenomatosis (54-year-old male, stage-IV)</td>
<td>21% (*99.7)</td>
<td>42% (*00.9), 47% (*01.2)</td>
</tr>
<tr>
<td>Gallbladder carcinoma (48-year-old female, stage II)</td>
<td>28% (*98.7)</td>
<td>40% (*98.8), 39% (*00.1), 48% (*01.5)</td>
</tr>
<tr>
<td>Bronchogenic cancer (58-year-old male, stage III-B)</td>
<td>26% (*99.5)</td>
<td>33% (*00.10), 39% (*01.11), 31% (*01.6)</td>
</tr>
<tr>
<td>Masto carcinoma (breast cancer) (43-year-old female, stage III)</td>
<td>30% (*98.10)</td>
<td>47% (*99.9), 53% (*00.12)</td>
</tr>
<tr>
<td>Parathyroid carcinoma (69-year-old male, stage III-B)</td>
<td>23% (*99.6)</td>
<td>38% (*99.9), 44% (*00.4), 62% (*01.6)</td>
</tr>
</tbody>
</table>

\(a\) NK activity (%) examined. Standard level of NK activity on human, 18—40%.

Fig. 4. IL-12 Release from RAW 264.7 Cells Stimulated with D-Fraction

RAW 264.7 cells (1×10⁶ cells/well in a 24-well plate) were stimulated for 18 h with D-Fraction. To remove any possible LPS contamination, D-Fraction was treated with polymyxin B (30 μg/ml) for 2 h before use. At the indicated time of incubation, IL-12 in the culture supernatants was measured using ELISA. Data are expressed as mean±S.E.M. of 3 experiments. *, \(p<0.05\) as compared with the basal (0 μg/ml of D-Fraction) (Scheffè’s F-test).

In conclusion, D-Fraction represents an important BRM for NK cells by enhancing IL-12 release from macrophages. In immunotherapy using D-Faction for cancer patients, NK cells are responsible for early anti-tumor responses, while both NK and T cells are responsible for long-term anti-tumor responses. Although the dependence of the immune system on NK cells requires further study, our results also indicate the possibility of immunotherapy by various agents, which enhance the activity of NK cells for cancer patients.

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REFERENCES