Polysaccharides from *Agaricus blazei* Stimulate Lymphocyte T-Cell Subsets in Mice

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Subset analysis of splenic lymphocytes using flow cytometry showed that the percentages of Thy1.2-(pan T-cells), L3T4-(CD4, helper T-cells), and Lyt2-(CD8, cytotoxic T-cells) positive cell populations were significantly increased in mice orally administered a hot water-soluble fraction from *Agaricus blazei* as compared with mice treated only with saline. 

The results seem to show that the polysaccharides from *Agaricus blazei* initiate anti-tumor activity through a modulation of the immune response system in tumor-bearing mice.

Although these experiments have shown the effects of polysaccharides as drugs on anti-tumor activity in tumor-bearing mice, we think that it is more important to clarify its effects on the immune response system in normal mice rather than tumor-bearing mice, considering these polysaccharides both as a prophylaxis and as a physiologically functional food. In this report, we will focus on the structure of polysaccharides from *Agaricus blazei* and their effects on T-cell subsets of spleen cells in normal mice.

**Materials and Methods**

*Animals.* Eight-week-old female C3H/He (Japan SL, Shizuoka) were used as sources for the splenic lymphocyte subsets.

*Materails.* The dry fruit bodies of *Agaricus blazei* were a gift from the Iwade Mushroom Institute (Mie, Japan).

**Extraction of the hot water-soluble fraction.** The dry fruit bodies of *Agaricus blazei* were homogenized with liquid nitrogen in a Waring blender and then lyophilized. The lyophilized mushrooms (10 g) were extracted 6 times with 80% ethanol (100 ml) at 80°C for 6 h. The residues after extraction with ethanol were extracted 4 times with hot water (200 ml) for 4 h and then filtered. The solution was lyophilized and is henceforth referred to as the hot water-soluble (HWS) fraction.

**Isolation of an active polysaccharide from the HWS fraction.** The HWS fraction (100 mg) from *Agaricus blazei* was dissolved in 100 ml water. The solution was added to 5% trichloroacetic acid to precipitate the proteins and then centrifuged for 15 min at 120 × g. The supernatant (FA) and the precipitate (FB) were dialyzed against distilled water through a cellulose tube. The residual solutions were concentrated to a small volume by evaporation and then lyophilized. The water solution of FA (5 mg) was submitted to anion exchange chromatography on a DEAE-Sepharose CL-6B column (2.6 × 34 cm) with a stepwise elution of 300 ml of 1/15 M phosphate buffer (pH 7.2), 400 ml of the same buffer containing 0.25 M NaCl, and then 300 ml of the same

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buffer containing 1 M NaCl. The active fraction was further separated by a Sepharose 6B column (1.6 × 94 cm) equilibrated with 1/15 M phosphate buffer (pH 7.2). The column was eluted with the same buffer.

Administration of sample. Each test sample was diluted to an adequate concentration with physiological saline, and a 0.2 ml-portion was injected directly into the stomachs of mice. Physiological saline was administered to the control group in the same manner.

Flow cytometry. The procedure for flow cytometry was that of Mizuno et al. with a slight modification. Murine spleen cells from C3H/He mice were suspended at a concentration of 1 × 10⁷/ml in the staining buffer (Dulbecco’s modified Eagle’s medium, 2% fetal bovine serum, 0.1% sodium azide) and incubated with either fluorescein isothiocyanate (FITC)-conjugated anti-Thy1.2 (pan T-cells, clone 30-H12), anti-Lyt2 (CD8; cytotoxic T-cells, clone 53-6.7), or phycoerythrin (PE)-conjugated anti-L3T4 (CD4; helper T-cells, clone GKL.5) monoclonal antibodies (Becton Dickinson Monoclonal Center, CA) for 30 min at 4°C to detect each subset of T-cells. The cell suspensions were then washed twice and resuspended in the staining buffer. The antibody-reacted cell suspensions were analyzed on a flow cytometer (EPICS PROFILE-II). The number of positive cells was calculated by integrating 10,000 cell-logarithmic fluorescence histograms above the channel in which no appreciable fluorescence was seen for the negative controls. Each value represents the mean ± S.D. of quintuplicate assays. The statistical significance of the differences was assessed using Student’s t test, with p < 0.05 and p < 0.01 as the criterion.

13C-NMR analysis. 13C-NMR spectra were measured with a Bruker AC250 spectrometer operating at 62.8 MHz in the pulse. All spectra were recorded for solution in D₂O by using 32 k data points and a spectra width of 19 kHz. 13C-chemical shifts are expressed in ppm downfield from external dioxane.

Results

The immunomodulating effects of 11 species of mushrooms were tested when the HWS fraction was injected directly into the peritoneal cavity. Seven of these species had immunomodulating effects (data not shown). Two of these 7 mushrooms, Agaricus blazei and Grifora frondosa, have already been observed to initiate antitumor activity against Sarcoma 180. To measure the influence of these 2 mushrooms as foods, the HWS fractions were injected directly into the stomachs of mice. The fractions from both mushrooms showed immunomodulating activity, as compared to the control groups (Table I). The HWS fraction from Agaricus blazei had the highest activity, and the percentages of Thy1.2, L3T4 (CD4), and Lyt2 (CD8) were 40.3, 25.0, and 14.3%, respectively. The FA fraction of Agaricus blazei showed a higher activity than the FB fraction (Table II). For example, with the percentage of L3T4 (CD4) and Lyt2 (CD8), the FA fraction was 30.6 and 17.3% with the increase of 23.5 and 26.0% as compared to the control, respectively. Therefore, the FA fraction was further fractionated by anion exchange chromatography on the DEAE-Sepharose CL-6B. The FA fraction consisted of polysaccharides and proteins at concentrations of 629.2 and 43.5 μg/mg, respectively (data not shown). This showed that the FA fraction was primarily a proteoglycan. As shown in Fig. 1, the FA fraction mainly produced 3 fractions, FA-I, II, and III, and the yields of each fraction based on weight were 66.6, 19.8, and 3.4%, respectively. The administration of FA-I showed an increase of 25.6, 28.4, and 34.3% in the population of Thy1.2, L3T4 (CD4), and Lyt2 (CD8) as compared to the administration of the crude polysaccharide from Agaricus blazei, respectively. FA-I, which showed immunomodulating activity and had the lowest protein content (Table III), was further fractionated by gel filtration on Sepharose 6B. One fraction was eluted at a total volume and another one, which was composed of proteoglycans was shown to have a molecular weight of 170,000 by using the following standard pullulan (Showa Denko K.K., Japan): P-400 (M.W.; 380,000), P-200 (186,000), P-100 (100,000), P-50 (48,000), P-20 (23,700) and P-10 (12,200).

The 13C-NMR data for this proteoglycan in D₂O has signals at δ98.8, 85.8, 80.2, 77.8, 74.9, 74.0, 71.6, 70.4, 69.2, and 61.4. An anomeric carbon signal can be observed at δ98.8, which corresponds to the α-glucopyranosyl residues. The signals at δ74.0, 74.9, 80.2, 71.6, and 69.2 correspond to C-2, 3, 4, and 5, and 6 of α-1,6-glucopyranosyl residues, respectively (Table IV), and the signals at δ77.8 and 61.4 can be assigned to the C-3 and 6 of α-1,4-glucopyranosyl residues, respectively. This immunomodulating polysaccharide is therefore presumed to be an α-1,4-glucan-linked α-1,6-
glucan in the main chain based on comparisons of other corresponding polysaccharide and monosaccharides found in the literature.\(^3\,14-16\)

**Discussion**

We observed an increase in the population of T-cell subsets after an oral administration of the polysaccharides from *Agaricus blazei* (α-1,6- and α-1,4-gluclan) into mice. The percentages of Thy1.2-, and Lyt2 (CD8)-positive cells among the splenic lymphocytes marked increased, as compared to the controls (Table III). The percentages of Thy1.2-, L3T4 (CD4)-, and Lyt2 (CD8)-positive cells were greater than those of the controls, with increases of 43.8, 37.2, and 50.0%, respectively. However, the complex of α-1,6- and α-1,4-gluclan did not have mitogenic activity by the MTT method using spleen cells or any changes of the population in spleen cells (data not shown), suggesting that the polysaccharides did not have any effects on the proliferation of cells. The L3T4/Lyt2 ratio decreased slightly, and the Lyt2/Thy1.2 ratio showed a tendency to increase in the group receiving the FA-I fraction. These results also suggest an increase especially in Lyt2 (CD8)-positive cells, which are considered to be markers of cytotoxic T-cells. We have also found that an intraperitoneal injection of the polysaccharides from *Agaricus blazei* increases the number of asialo GM1-positive cells (data not shown), which are considered to be markers of natural killer T-cells. Thus, this polysaccharide appears to have an effect on the changing pattern of splenic lymphocyte subsets, particularly cytotoxic or natural killer cells.

Polysaccharides from the mushrooms have been the focus of much research because they have shown anti-tumor activity against mice-implanted tumors. Recently, Mizuno et al.\(^3\) have reported that polysaccharides from *Agaricus blazei* have anti-tumor activity and that one of these polysaccharides has a 1,6- and 1,4-gluclan complex as the main component, similar to the immunomodulating polysaccharide investigated in our current study (Table IV). It has also been reported that the percentages of splenic Thy1.2-, L3T4-, and asialo GM1-positive cells were significantly higher than in tumor-bearing mice treated with saline when polysaccharides from *Agaricus blazei* (β-1,6-gluclan) were administered.\(^10\) We have demonstrated that the administration of these polysaccharides (α-1,6- and α-1,4-gluclan complex) increases the population of the splenic lymphocyte subsets even in normal mice. As the number of cytotoxic and natural killer T-cells are particularly increased in the presence of this polysaccharide, there may be a stronger possibility that the cancer cells are attacked by these lymphocytes. This seems to suggest that the polysaccharides from *Agaricus blazei* may be important both as a prophylaxis against cancer and as a functional food.

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**Table III.** Cell Population of Splenic T-Lymphocyte Subsets after Oral Administration of Three Fractions Separated by DEAE-Sepharose CL-6B and the Contents of Sugar and Protein in Each Fraction.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Percentage of positive cells (% ± S.D.)</th>
<th>Contents (μg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thy1.2</td>
<td>L3T4</td>
</tr>
<tr>
<td>FA-I</td>
<td>50.6±4.0*</td>
<td>32.1±3.2*</td>
</tr>
<tr>
<td>FA-II</td>
<td>51.8±2.9*</td>
<td>33.0±5.6*</td>
</tr>
<tr>
<td>FA-III</td>
<td>39.7±6.8</td>
<td>25.9±4.0</td>
</tr>
<tr>
<td>Control</td>
<td>35.2±2.2</td>
<td>23.4±1.9</td>
</tr>
</tbody>
</table>

Significant differences from the control group: *p < 0.01.

The contents of polysaccharides and proteins were calculated as glucose and bovine serum albumin, respectively. The experiment was done as described in Table I.
Immunomodulating Activity of *Agaricus blazei*

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**References**