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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. ^{13}C-NMR data indicates that the main component in the active polysaccharide is the complex of α-1,6- and α-1,4-glucan, which had already been shown to have anti-tumor activity against Sarcoma 180. It seems that the polysaccharide from *Agaricus blazei* may be an effective prophylactic, protecting humans against cancer by stimulating lymphocytes such as cytotoxic T-cells.

Key words: *Agaricus blazei*; T-cell subsets; flow cytometry; immunomodulating activity; mushrooms

Mushrooms have recently become attractive as a functional food and a source for the development of drugs. Many investigators have isolated and identified anti-tumor polysaccharides from mushrooms.[1-5] The anti-tumor activity of these polysaccharides is caused by the potentiation of the immune response, which involves lymphocyte activation.[2,6] Hamuro *et al.*[7,8] have reported that lentinan, a β (1→3) glucan, mediates the augmentation of alloreactive murine cytotoxic T-lymphocytes and increases the generation of cytotoxic T lymphocytes in spleen cells. Sakagami *et al.*[9] have also reported that schizophyllan, a β (1→6) branched β (1→3) glucan, activates macrophages, followed by the potentiation of killer T-cells through cytokine mediation. It therefore seems that the anti-tumor polysaccharides commonly show activity by stimulating T-cell subsets in spleen cells.

Mizuno *et al.*[4,5] have reported that polysaccharides from *Agaricus blazei* have anti-tumor activity against Sarcoma 180-bearing mice. The structure of the polysaccharides includes β-1,6-glucopyranosyl residues as a backbone.[3] Ito *et al.*[10] have reported that macrophage activation and an alteration of the third component of complement is necessary for the induction of an anti-tumor effect when polysaccharides from *Agaricus blazei* are injected intraperitoneally into a mouse-implanted tumor. In addition, it has been reported that the percentages of splenic Thy1.2-, L3T4-, and asialo GM1-positive cells in the T-cell subsets were significantly higher than those of tumor-bearing mice treated with saline.[11] This suggests that the changing pattern of the Thy1.2-, L3T4-, and asialo GM1-positive cells correlates with the

anti-tumor effects of the polysaccharides in *Agaricus blazei*. These 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 SLC, Shizuoka) were used as sources for the splenic lymphocyte subsets.

Materials. 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 Warning 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

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.*[12] with a slight modification. Murine spleen cells from C3H/He mice were suspended at a concentration of 1×10^6/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; GK1.5) 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 Brucker AC250 spectrometer operating at 62.8 MHz in the pulse. All spectra were recorded for solution in D_2O by using 32 k data points and a spectra width of 19 kMHz. 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 *Glifora frondosa*, have already been observed to initiate anti-tumor activity against Sarcoma 180.[1,3-5] 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

Table I. Cell Population of Splenic T-Lymphocyte Subsets After Oral Administration of the HWS Fraction from *Agaricus blazei*, *Grifora frondosa*, and the *Grifora frondosa* Mutant.

Mushrooms	Percentage of positive cells (%, mean ±S.D.)		
	Thy1.2	L3T4	Lyt2
Agaricus blazei	40.3±7.8**	25.0±2.9**	14.3±2.3**
Grifora frondosa	35.7±5.6	21.9±4.9*	13.7±2.5
Grifora frondosa mutant	39.5±4.9**	24.5±3.0*	14.3±2.2**
Control	29.4±7.8	19.4±5.4	10.3±3.4

Significant differences from the control group: *, $p < 0.05$: **, $p < 0.01$.

Each HWS fraction was given *p.o.* every 3 days for 4 weeks at as dose of 100 mg/kg mouse weight. Each group consisted of 5 mice.

Table II. Cell Population of Splenic T-Lymphocyte Subsets after Oral Administration of FA and FB Fractions.

Fractions	Percentage of positive cells (%, mean ±S.D.)		
	Thy1.2	L3T4	Lyt2
FA fraction	47.5±4.9*	30.6±2.5*	17.3±1.6*
FB fraction	42.2±4.0	27.6±3.1	14.8±1.8
Control	35.2±2.2	23.4±1.9	12.8±1.8

Significant differences from the control group: *, $p < 0.01$.
The experimental conditions were the same as those described in Table I.

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 increases 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 D_2O 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.[13] The signals at δ74.0, 74.9, 80.2, 71.6, and 69.2 correspond to C-2, 3, 4, 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.[14] This immunomodulating polysaccharide is therefore presumed to be an α-1,4-glucan-linked α-1,6-
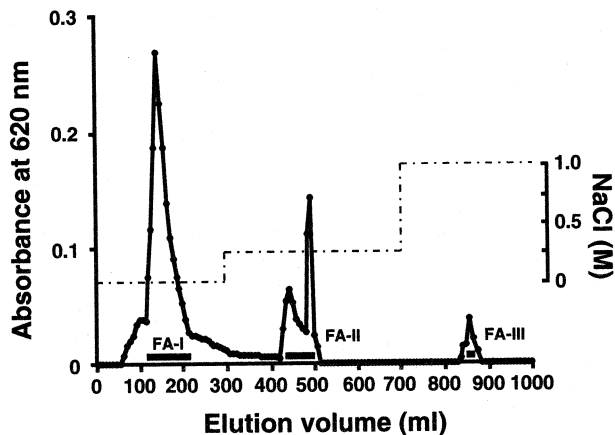
Fig. 1. Elution Profile of the FA Fraction on a Column of DEAE-Sepharose.

The sugar contents of each fraction were measured at 620 nm by the anthron-H_2SO_4 method. Each bar shows the range of fractions collected for the immunomodulating assay.

Table IV. The ^{13}C-NMR Chemical Shift of the Polysaccharide from *Agaricus blazei* Compared to the Authentic Monosaccharide or Polysaccharide.

	Chemical shift (ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
Active polysacchride	98.8	74.0	74.9	80.2	71.6	69.2
α-Glucose*	92.9	72.5	73.8	70.6	72.3	61.6
β-Glucose*	96.7	75.1	76.7	70.6	76.8	61.7
Laminarin[†]	102.9	72.8	86.2	68.3	76.2	60.8
FA-1-a-α[‡]	100.9	74.2	75.2	80.2	72.0	69.0
FIII-2b[¶]	106.5	76.6	80.1	73.2	78.6	72.2

*: The data cited from Bock and Pedersen[15].
[†]: The data cited from Muller et al.[16].
[‡]: The date cited from Mizuno et al.[14].
[¶]: The data cited from Kawagishi et al.[3].

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-glucan) into mice. The percentages of Thy1.2-, and Lyt2 (CD8)-positive cells among the splenic lymphocytes markedly 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-glucan 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.[14] have reported that polysaccharides from *Agaricus blazei* have anti-tumor activity and that one of these polysaccharides has a 1,6- and 1,4-α-glucan 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-glucan) were administered.[11] We have demonstrated that the administration of these polysaccharides (α-1,6- and α-1,4-glucan 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.

Acknowledgments

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.

Fractions	Percentage of positive cells (%, mean ±S.D.)			Contents (μg/mg)	
	Thy1.2	L3T4	Lyt2	Polysaccharides	Proteins
FA-I	50.6±4.0*	32.1±3.2*	19.2±2.1*	731.5	2.3
FA-II	51.8±9.1*	33.0±5.6*	19.8±3.6*	778.6	7.7
FA-III	39.7±6.8	25.9±4.0	14.9±2.6	134.1	16.9
Control	35.2±2.2	23.4±1.9	12.8±2.6	—	—

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.

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