# Polysaccharides in Fungi. XXX.[1] Antitumor and Immunomodulating Activities of Two Polysaccharides from the Fruiting Bodies of *Armillariella tabescens*

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**The effects of two polysaccharides, AT-HW and AT-AL obtained from the fruiting bodies of *Armillariella tabescens* on murine sarcoma 180 tumor and peritoneal macrophages were examined at intraperitoneal administration. AT-HW from the hot-water extract and AT-AL from the alkaline extract significantly inhibited the tumor, and the results of different administration schedule and phagocytic system blockade suggested that the mechanism of AT-AL differed from that of AT-HW and branched (1→3)-β-D-glucans. AT-HW and AT-AL showed reticuloendothelial system-potentiating activity, increased the number of peritoneal exudate cells, activated on macrophages (acid phosphatase activity, glucose consumption, superoxide anion production), and enhanced mitogenic reaction, although AT-HW did not produce superoxide anion *in vitro*.**

**Keywords** *Armillariella tabescens*; polysaccharide; antitumor activity; macrophage; immunomodulator

*Armillariella tabescens* (FR.) SING. (Tricholomataceae) is an edible mushroom. We have isolated two polysaccharides (AT-HW and AT-AL) from the fruiting bodies harvested in Japan. There are many studies[2-4] on the antitumor and immunomodulating activities of polysaccharides such as β-(1→6)-branched (1→3)-β-D-glucans from fungi. As described in the previous paper,[1] AT-HW obtained from hot-water extract is a water-soluble heteroglycan (composed of D-glucose, D-galactose, D-mannose, and D-fucose) containing small amounts of peptide moieties and AT-AL obtained from the alkaline extract is a water-insoluble, (1→3)-α-D-glucan containing small amounts of other sugar residues. Since two kinds of the polysaccharides are attractive materials in view of biological response modifiers (BRMs), we examined their antitumor and immunomodulating activities in mice, in particular by the use of murine peritoneal exudate macrophages, by intraperitoneal (i.p.) administration.

The antitumor activity on sarcoma 180 and reticuloendothelial system-potentiating activity were tested. Effects on murine peritoneal macrophages were investigated by some experiments, and mitogen activity was assayed. Thus, the activities of AT-HW and AT-AL were investigated and compared with each other.

## Materials and Methods

**Mice** Male, ddY mice were purchased from Japan SLC, Inc. (Shizuoka, Japan), and used at 5- to 7-weeks-old.

**Assay of Antitumor Activity** Sarcoma 180 ascite cells ($1.5 \times 10^6$) were inoculated subcutaneously into the right groin of mice (5 weeks). Each sample was dissolved or suspended in physiological saline, and administered i.p. daily for 5 d (days +2 to +6 or days +7 to +11) after tumor transplantation (day 0). After 30 d, the mice were sacrificed, and the tumors were extirpated and weighed. The inhibition ratios (%) were given by $[(A-B)/A] \times 100$, where $A$ is the average tumor weight of the control group, and $B$ is that of the test group. Furthermore, to investigate the effect of the phagocytic system blockade on the activity of the polysaccharides, carrageenan (Sigma Chemical Co.) (4 mg/mouse) for 3 d (days −4, −2, 0) or trypan blue (Hayashi Pure Chemical Industries) (2 mg/mouse) once (day 0) before tumor transplantation (day 0) was injected i.p. to mice. The mice were administered i.p. with each polysaccharide (300 μg/mouse/d) daily for 5 d (days +1 to +5) after tumor transplantation. The antitumor activity was evaluated at 30 d after the transplantation as described above.

**Carbon Clearance Test** The test was examined in mice, as described previously.[5] Zymosan (Tokyo Kasei Kogyo Co.) was used as a positive control. Mice (5 weeks) were injected with colloidal carbon at a dose of 0.2 ml/mouse *via* the tail vein at 48 h after i.p. injection of each polysaccharide (10 mg/kg body weight). The clearance rate was expressed as the half-life period of carbon in the blood ($t_{1/2}$, min), calculated by $[\ln 2(t_2-t_1)]/(\ln OD_1 - \ln OD_2)$, where $OD_1$ and $OD_2$ are optical densities at times $t_1$ and $t_2$, respectively.

**The Number of Peritoneal Exudate Cells** Mice (6 weeks) were injected i.p. with each polysaccharide or saline (control) for 3 d. The mice were sacrificed at 24 h after the last injection, and peritoneal exudate cells (PEC) were obtained with Hanks' solution (Nissui Seiyaku Co.) from the peritoneal cavity of the mice. The cells were stained with Türks' reagent, and the number was counted with a hemocytometer.

**Assay of Acid Phosphatase Activity in Macrophages** Mice (6 weeks) were administered i.p. with AT-HW or AT-AL or zymosan (positive control) or saline (control) daily for 3 d. At 24 h after completion of administration, PEC were collected from the mice by washing with Hanks' solution. The cells were washed twice with RPMI 1640 medium (Nissui Seiyaku Co.) containing 5 mM *N'*-hydroxyethylpiperazine-*N'*-2-ethansulfonate (Nacalai Tesque, Inc.), 100 U/ml penicillin, 100 μg/ml streptomycin (Meiji Seika Co.), and 10% heat-inactivated fetal calf serum (FCS) (M.A. Bioproducts). These cells suspended in RPMI 1640 medium were placed on a plate (Nunc, Inc.), cultured at 37 °C for 1 h in a $CO_2$ incubator, and non-adherent cells were removed by washing with Hanks' solution. The adherent cells were collected with a rubber policeman, washed twice, then resuspended in RPMI 1640 medium. The cells ($1 \times 10^5$) suspension in a test tube was centrifuged, and the precipitate was added 0.1 ml of 0.1% Triton X-100 (Kishida Chemical Co.), 0.5 ml of 1-nitrophenyl phosphate as a susbstrate, and 0.4 ml of 0.1 M citrate buffer (pH 5.0). The mixture was incubated at 37 °C for 1 h, and 1 ml of 0.2 M borate buffer (pH 9.8) was added, then the optical density at 405 nm was measured.

**Effect on Macrophages Induced with Liquid Paraffin** Mice (6 weeks) were injected i.p. once with liquid paraffin (Nacalai Tesque) (0.5 ml). Four days later, PEC were similarly collected, $3.3 \times 10^5$ of the cells per well of a 96-well flat bottom plate were cultured in a $CO_2$ incubator. The adherent cells were suspended in RPMI 1640 medium containing 10% FCS, and each polysaccharide or lipopolysaccharide (LPS) (Difco Laboratories) was added to each well. The enzyme activities in the supernatant and the solubilized cells were measured with a microplate reader (Corona Electric Co.).

**Assay of Glucose Consumption Activity** Macrophages were obtained from PEC induced with liquid paraffin as described above. Each sample or schizophyllan (a kind gift from Kaken Chemical Co.) was added to the macrophage supernatant, and the mixtures were cultured at 37 °C in a $CO_2$ incubator. After 24 and 72 h, glucose remaining in the macrophage culture supernatant was determined by the use of Glucose B-test Wako (Wako Pure Chemical Industries) based on the glucose-oxidase method. Glucose consumption (%) was obtained by the equation of $(1-S/C) \times 100$, where $S$ is the glucose content in the culture with sample, $C$ is the glucose content without sample.

**Measurement of Superoxide Anion (SOA)** Each Sample was administered i.p. daily for 3 d, and peritoneal macrophages on 24 h after completion of administration were obtained as described above. Nitro blue tetrazolium (NBT) (Nacalai Tesque) (2 mg/ml) dissolved in RPMI 1640 medium was pipetted into each well of macrophages, and the mixture with

or without opsonized zymosan[6] (10 mg/ml) was incubated at 37 °C for 30 min. The reaction mixture was fixed with methanol, and the optical density of the solution dissolved in KOH/dimethyl sulfoxide was measured at 630 nm with a microplate reader. To investigate the direct effect on macrophages, paraffin-induced macrophages were incubated with samples for 24 h, and the amount of SOA was determined by the method of NBT.[7]

**Assay of Mitogen Activity** The spleen cells obtained from mice (6 weeks) were untangled, then dispersed in RPMI 1640 medium containing 10% FCS. After the suspension (100 μl) and each sample (25 μl) were cultured for 72 h in 96-well plates in a $CO_2$ incubator, 20 μl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide (MTT)[8] (Dojin Laboratories) (5 mg/ml) was added and the mixture was incubated for 5 h. The reaction was stopped by the addition of 20% sodium laurylsulfate/HCl. Standing over 4 h, the optical density at 550 nm was measured with a microplate reader. A positive control used Concanavalin A (Con A) (Pharmacia LKB Biotechnology).

**Endospecy Test (ES-Test)** The amount of LPS in samples was determined by the colorimetric Limulus test, ES-test[9] (Seikagaku Kogyo) using endotoxin (LPS) from *E. coli* 0111:B4 as a reference standard.

**Statistics** The significance of differences between means was evaluated by the use of Student's *t* test.

## Results

**Evaluation of Antitumor Activity** The antitumor activity of AT-HW and AT-AL on murine sarcoma 180 were examined at different schedules of i.p. administration. As shown in Table I, both polysaccharides (300 μg/mouse/d) were effective at early administration for 5 d (+2 to +6) after tumor transplantation (0). AT-AL from the alkaline extract was less active at a later administration for 5 d (+7 to +11), although AT-HW from the hot-water extract exhibited significant activity. Since the phagocytic system is important for the antitumor activity of polysaccharides,[10] the effect of phagocytic system blocking reagents, *i.e.*, carrageenan[11] and trypan blue,[12] on the activities of AT-HW and AT-AL was examined. The results are summarized in Table II. The antitumor activity of AT-HW was suppressed by the carrageenan, while that of AT-AL was not suppressed. An inhibitor of lysosomal enzymes in the phagocytic system, trypan blue which diminished the antitumor activities of mannan[13] and branched (1→3)-β-D-glucan,[10,14] also decreased the activity of both polysaccharides.

TABLE I. Effect of Various Schedules for AT-HW and AT-AL Administration on the Inhibition of Sarcoma 180 Growth in Mice

| Sample | Days of therapy | No. of mice | Tumor weight (g, mean ± S.E.) | Inhibition ratio | $p^{a)}$ |
|---|---|---|---|---|---|
| AT-HW | +2—+6 | 5 | 1.40±0.66 | 77 | $p<0.005$ |
| | +7—+11 | 5 | 0.96±0.66 | 85 | $p<0.005$ |
| AT-AL | +2—+6 | 5 | 1.80±1.03 | 71 | $p<0.05$ |
| | +7—+11 | 5 | 3.42±0.85 | 45 | n.s.[b)] |
| Control | — | 10 | 6.17±1.04 | — | |

*a*) Significant difference from the control. *b*) Not significant.

TABLE II. Effect of Phagocytic System Blocking Reagents on the Antitumor Activity of AT-HW and AT-AL

| Group | Sample[a)] | Treatment[b)] | No. of mice | Tumor weight (g, mean ± S.E.) | Inhibition ratio (%) | $p^{c)}$ |
|---|---|---|---|---|---|---|
| 1 | Control | — | 5 | 9.30±2.50 | — | — |
| 2 | AT-HW | — | 7 | 1.55±0.74 | 83 | $p<0.05$ |
| 3 | AT-AL | — | 6 | 2.09±1.25 | 78 | $p<0.05$ |
| 4 | Control | Carrageenan | 5 | 5.02±1.24 | 46 | — |
| 5 | AT-HW | Carrageenan | 7 | 3.78±0.96 | 59 | n.s. |
| 6 | AT-AL | Carrageenan | 5 | 1.97±0.89 | 79 | $p<0.05$ |
| 7 | Control | Trypan blue | 8 | 9.12±2.45 | 2 | — |
| 8 | AT-HW | Trypan blue | 9 | 3.72±1.94 | 60 | n.s. |
| 9 | AT-AL | Trypan blue | 9 | 6.01±2.26 | 35 | n.s. |

*a*) Each sample (300 μg/mouse) was administered by i.p. injection for successive days (days +1—+5). *b*) Carrageenan (4 mg/mouse) was i.p. administered on days −4, −2, 0, and trypan blue (2 mg/mouse) was i.p. administered on day 0. *c*) Significant difference from the control (groups 1, 4, 7).

**Effect on the Reticuloendothelial System** Phagocytic activity of the polysaccharides was examined *in vivo* by the carbon clearance test.[15] As shown in Fig. 1, AT-HW enhanced the murine reticuloendothelial system, while the effect of AT-AL had a positive, but not significant tendency, at a single dose of 10 mg/kg.

**Examination of the Effect on Murine Peritoneal Macrophages** AT-HW at doses of 300 μg/mouse/d and AT-AL at doses of 100 or 300 μg/mouse/d remarkably increased the number of PEC, although AT-HW at doses of 100 μg/mouse/d did not increase the number, compared with control (saline) as shown in Fig. 2. The activity may also be related to the solubility of the sample, *i.e.*, AT-HW is soluble and AT-AL is insoluble in water because the insoluble material such as zymosan is more recognized by the phagocyte.

The effect of the polysaccharides on macrophages *in vivo* was evaluated by measurement of the lysosomal enzyme

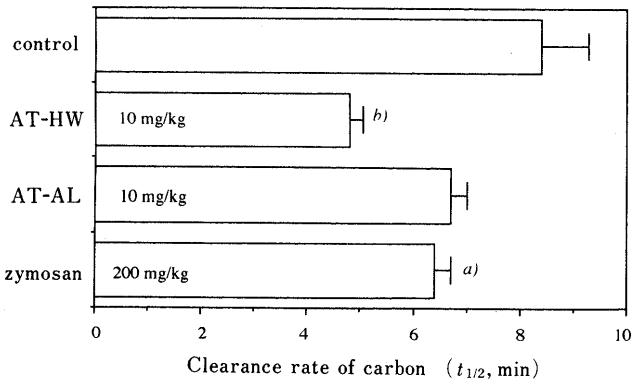


Fig. 1. Effect of AT-HW and AT-AL on Clearance Rate of Carbon Particles from Blood Circulation in ddY Mice

Results are expressed as the arithmetic mean + S.E. of mice. Significant difference from the control, *a*) $p<0.05$, *b*) $p<0.01$.

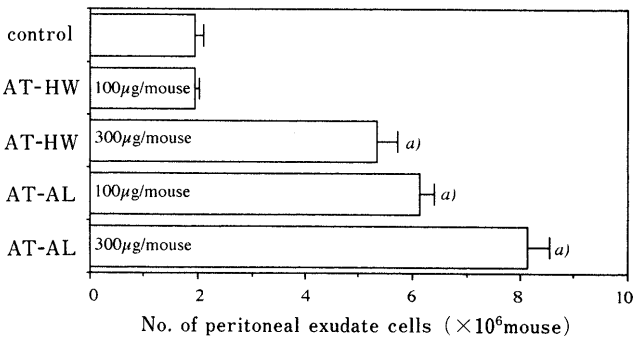


Fig. 2. Effect of AT-HW and AT-AL on Number of Peritoneal Exudate Cells

Each sample was administered by i.p. injection for 3 consecutive days. The day after the final injection, peritoneal exudate cells were isolated from the peritoneal cavity by lavage with Hanks' solution, and counted by a dye exclusion method with Türks' solution. All values are expressed as the arithmetic mean + S.E. of 4 mice. Significant difference from the control, *a*) $p<0.001$.

Table III. Effect of AT-HW and AT-AL on Acid Phosphatase Activity in Macrophages

| Sample[a] | Dose (μg) | Enzyme activity[b] (±S.E.) | $p$[c] |
|---|---|---|---|
| AT-HW | 50 | 57.1±8.49 | $p<0.01$ |
| | 100 | 90.6±5.08 | $p<0.001$ |
| AT-AL | 50 | 30.6±3.45 | $p<0.001$ |
| | 100 | 43.4±3.34 | $p<0.001$ |
| Zymosan | 100 | 79.7±6.04 | $p<0.001$ |
| Control | — | 11.1±1.42 | — |

*a*) Each sample was administered i.p. into ddY mice for 3 consecutive days. *b*) *p*-Nitrophenol (nmol) per $1\times10^5$ macrophages per 60 min. *c*) Significant difference from the control.

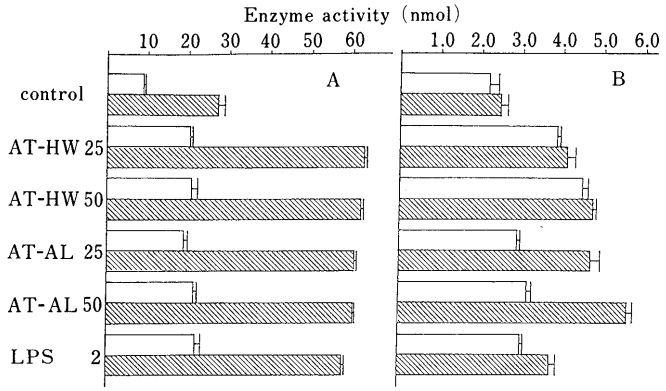


Fig. 3. Lysosomal Enzyme Production by Macrophages Incubated with AT-HW and AT-AL

The production of acid phosphatase by macrophage cultured for 24 h (open columns) and 72 h (hatched columns) at 37 °C with each sample was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl phosphate in cell lysate (A) and culture supernatant (B). The macrophages ($3\times10^5$/well) were cultured in 200 μl of medium with each sample. The Concentration of AT-HW and AT-AL was 25 and 50 μg/culture, and that of LPS was 2 μg/culture.

(acid phosphatase) activity. AT-HW and AT-AL (50 or 100 μg/mouse/d) enhanced the activity by i.p. administration for 3 d, and the enhanced activity of AT-AL was about twice that of AT-HW (Table III). Since it is known that the enzyme activity is enhanced by the C3b component of the alternative complement pathway,[16] the polysaccharides may be able to activate the pathway.

The effect on macrophages induced with liquid paraffin was evaluated as follows: Macrophages obtained by stimulation of liquid paraffin were directly added to each polysaccharide (25 or 50 μg/culture), and cultured for 24 and 72 h, then the activity of acid phosphatase in both cell lysate and culture supernatant was measured. The positive effect of AT-HW and AT-AL *in vitro* on paraffin-induced peritoneal macrophages was significantly observed in both fractions as shown in Fig. 3. It has been reported that the lysosomal enzyme in macrophages participates in the antitumor activity,[16] therefore, the results indicate that the antitumor activity of AT-HW and AT-AL on sarcoma 180 is closely related to the enzyme activation in macrophages.

The effect of the polysaccharides on macrophages induced with liquid paraffin was evaluated by measuring the glucose consumption activity. As shown in Table IV, AT-HW and AT-AL (25 or 50 μg/culture) significantly enhanced the glucose consumption. AT-HW and AT-AL more consumed the glucose in macrophages than schizophyllan. The results suggest that the polysaccharides stimulated macrophages at an early stage, but it is obscure whether the effect is lasting or not until the last stage, in the cascade of macrophage activation.

Table IV. Effect of AT-HW and AT-AL on Glucose Consumption by Macrophages[a]

| Sample | Dose (μg) | Consumption % (±S.E.) | $p$[b] | Ratio |
|---|---|---|---|---|
| AT-HW | 25 | 10.8±1.59 | $p<0.05$ | 1.94 |
| | 50 | 14.8±0.44 | $p<0.001$ | 2.66 |
| AT-AL | 25 | 12.3±1.58 | $p<0.05$ | 2.21 |
| | 50 | 13.3±0.83 | $p<0.001$ | 2.38 |
| Schizophyllan | 25 | 6.6±0.76 | n.s.[c] | 1.19 |
| | 50 | 8.3±0.24 | $p<0.005$ | 1.59 |
| Control | — | 5.6±0.32 | — | 1.00 |

*a*) The glucose content in culture supernatant obtained from the wells after incubation for 24 h at 37 °C with samples was assessed by a Glucose B-test Wako. The macrophages ($2\times10^5$/well) were cultured in 200 μl of medium with various additives. *b*) Significant difference from the control. *c*) Not significant.

Table V. NBT Reduction by Resident, AT-HW- and AT-AL-Induced Adherent Peritoneal Cells[a]

| Cells | NBT reduction (Mean±S.E.) | |
|---|---|---|
| | Opsonized zymosan (−) | Opsonized zymosan (+) |
| Resident | 0.084±0.004 (1.00) | 0.230±0.003 (1.00) |
| AT-HW-Induced | 0.221±0.011[c] (2.63) | 0.266±0.006[c] (1.16) |
| AT-AL-Induced | 0.208±0.003[c] (2.48) | 0.243±0.004[b] (1.06) |

*a*) Adherent peritoneal cells (macrophages) were prepared on a 96 well flat-bottomed plate, and cells were reacted with NBT in the presence or absence of opsonized zymosan. Formazan produced by reduction of NBT by active oxygen species was solubilized by KOH/DMSO treatment and absorbance at 630 nm was determined. Significant difference from control, *b*) $p<0.01$, *c*) $p<0.001$.

Table VI. Direct Effect of AT-HW and AT-AL on NBT Reduction by Paraffin-Induced Macrophages[a]

| Sample | μg/culture | NBT reduction (Mean±S.E.) | |
|---|---|---|---|
| | | Opsonized zymosan (−) | Opsonized zymosan (+) |
| Control | — | 0.164±0.005 (1.00) | 0.268±0.012 (1.00) |
| AT-HW | 25 | 0.157±0.010 (0.96) | 0.195±0.007 (0.73) |
| | 50 | 0.176±0.009 (1.07) | 0.232±0.021 (0.87) |
| AT-AL | 25 | 0.323±0.006[c] (1.97) | 0.325±0.006[b] (1.21) |
| | 50 | 0.382±0.020[c] (2.33) | 0.369±0.010[c] (1.34) |

*a*) Paraffin-induced macrophages ($4\times10^5$/well) were cultured in 200 μl of medium with various additives for 24 h, and the cells were reacted with NBT. Formasan was solubilized and absorbance at 630 nm was determined. Significant difference from the control, *b*) $p<0.01$, *c*) $p<0.001$.

The level of SOA produced by macrophages induced with polysaccharides was measured by the NBT reduction method. In comparison with resident peritoneal cells, the cells induced with AT-HW and AT-AL slightly produced SOA with opsonized zymosan, and significantly produced

TABLE VII. Effect of AT-HW and AT-AL on MTT Dye Reduction of Spleen Cells[a]

| Sample | μg/culture | Absorbance (550 nm)[b] | Stimulation index[c] |
|---|---|---|---|
| AT-HW | 0.01 | 0.387±0.012 | 1.2 |
| | 0.1 | 0.408±0.011 | 1.2 |
| | 1 | 0.435±0.002 | 1.3 |
| | 10 | 0.387±0.008 | 1.2 |
| | 100 | 0.470±0.004 | 1.4 |
| AT-AL | 0.01 | 0.376±0.018 | 1.1 |
| | 0.1 | 0.362±0.014 | 1.1 |
| | 1 | 0.391±0.017 | 1.2 |
| | 10 | 0.415±0.009 | 1.3 |
| | 100 | 0.367±0.013 | 1.1 |
| Con A | 0.3 | 0.450±0.006 | 1.4 |
| Control | | 0.330±0.006 | 1.0 |

*a*) $6.3 \times 10^5$ spleen cells from ddY mice were incubated with each sample for 72 h then assessed by MTT colormetric assay. *b*) Arithmetic mean absorbance ± S.E. of 4 replicate cultures. *c*) Mean absorbance in experimental culture/mean absorbance in control culture.

without opsonized zymosan, as shown in Table V. Furthermore, the effect of the polysaccharides, *in vitro*, on the SOA production of macrophages induced with liquid paraffin was measured by a similar method. As shown in Table VI, AT-AL (25 or 50 μg/culture) significantly produced SOA with and without opsonized zymosan, while AT-HW did not affect the production. The results suggested that AT-AL activated the pentose-phosphate pathway, and the resulting reduced nicotineamide adenine dinucleotide phosphate (NADPH) produced SOA, but an effect of AT-HW was different from that of AT-AL.

**Mitogen Activity** The mitogen activity of the polysaccharides was investigated in a culture of spleen cells by the MTT method using Con A as a positive control. Both samples (0.01—100 μg/culture) significantly affected the multiplying reaction of the lymphocyte (Table VII). As it has been reported[8] that the value of Con A was given to be 5—6 times by the $^3$H-thymidine method and about 1.5 times by the MTT method, in comparison with the control in the mitogenic activity test, the mitogenic activity of AT-HW and AT-AL may show higher values by the isotropic method.

**Endotoxin Contamination** Endotoxin contamination of AT-HW and AT-AL was checked by the colorimetric Limulus test, ES-test. The values of AT-HW and AT-AL were 423 ng/mg and 5 ng/mg of reference *E. coli* LPS, respectively.

## Discussion

We have isolated two kinds of polysaccharides, AT-HW and AT-AL, from the fruiting bodies of *A. tabescens*.[1] AT-HW obtained from the hot-water extract is mainly composed of β-(1→6)-linked D-glucopyranosy and D-galactopyranosyl residues, β-(1→3)-linked D-galactopyranosyl residues, their branched residues, and terminal (fucose, mannose and glucose) residues, and also contains about 13% of peptide moieties. On the other hand, AT-AL obtained from the alkaline extract is (1→3)-α-D-glucan and free from nitrogen. The molecular weights of AT-HW and AT-AL have similar values, about 100000.

By i.p. administration of AT-HW and AT-AL in mice, we studied the antitumor activity against sarcoma 180, and the immunomodulating activity focused on macrophage activation because macrophages have an important role in self-defense mechanisms. Both samples significantly inhibited the tumor growth, and the studies on the administration at different schedules and phagocytic system blocking reagents suggest that the active mechanism of AT-HW is similar to that of 6-branched (1→3)-β-D-glucans such as lentinan from *Lentinus edodes*[2,14] or schizophyllan from *Schizophyllum commune*,[17] and indicate that the activities of AT-HW and AT-AL require the partition of the phagocytic system and macrophages. However, AT-AL showed different results from AT-HW in that the antitumor activity was less effective by the later administration, unsuppressed by carrageenan treatment, and the reticuloendothelial system-potentiating activation was weaker. AT-AL increased the number of murine peritoneal exudate cells more than AT-HW, which can be assumed to be largely due to the solubility and/or chemical structure of the sample.

Macrophage activation in murine peritoneal exudate macrophages was evaluated by lysosomal enzyme (acid phosphatase) activation and SOA production, *in vivo* and *in vitro*, and by glucose consumption. Both samples significantly enhanced the above phenomena except for the SOA production of AT-HW *in vitro*. The results of lysosomal enzyme activation *in vivo* and in paraffin-induced macrophage suggested the participation of the alternative complement pathway. AT-HW enhanced glucose consumption, and produced SOA *in vivo*, but not *in vitro*. The production *in vivo* may be accelerated by some signals mediated by other cells *e.g.*, T-cells. Thus, it is suggested that AT-HW has a similar immunomodulating mechanism to branched (1→3)-β-D-glucan, *e.g.*, lentinan, and the mechanism of AT-HW could be partially attributed to a similar structure on the part of AT-HW.

Since endotoxin (LPS) shows many biological activities[18] such as macrophage activation, its contamination is important in the studies on immunostimulating compounds. The amount of LPS in AT-HW and AT-AL was determined by the Endospecy test. This test was recently established, and is unaffected by (1→3)-β-D-glucans. Limulus activity corresponding to 423 ng of a reference *E. coli.* LPS was detected in 1 mg of AT-HW and hardly detectable in AT-AL. The content of AT-HW could never result in significant biological activities, although LPS in AT-HW at a higher dose may slightly affect the mitogen activity which was significantly observed in AT-HW and AT-AL by the MTT method.

The foregoing results suggest that AT-HW affects other cells such as T-cells or alternative complement pathway, in addition to macrophages, while AT-AL acts primarily on macrophages, and the activated macrophages produce SOA and activate lysosomal enzyme. Thus, two polysaccharides showed different results in some activities of this experiment. AT-HW is a water-soluble heteroglycan containing small amounts of peptide moieties, and AT-AL is a water-insoluble (1→3)-α-D-glucan. The studies would give interesting information on the structure–activity relationship of polysaccharide. The studies on cytokine, natural killer cells, *etc.*, directly related to the antitumor activity at the next step in the immunological network will

be on reported in the near future.

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