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Oncostatic and immunomodulatory effects of a glycoprotein fraction from water extract of abalone, *Haliotis discus hannai*

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Summary. The liquid from heat-treatment of an abalone, *Haliotis discus hannai,* which is normally discarded as waste, was found to contain a new glycoprotein antineoplastic agent. A fraction of the liquid obtained from chromatography that was 22% carbohydrate and 44% protein was injected locally or systematically into ICR mice or BALB/c mice inoculated s.c. with allogeneic sarcoma 180 or syngeneic Meth-A fibrosarcoma, and growth of the tumors was strongly inhibited. There was an optimum dose range for the inhibition of the growth of sarcoma 180, and optimum timing. The fraction did not have antitumor activity in T cell-deficient nude mice (CD-1 nu/nu or BALB/c nu/nu mice), and administration of carrageenan in vivo decreased its activity in ICR mice. This fraction activated the cytostatic activity of peritoneal and alveolar macrophages in vivo. These results suggest that the antitumor activity is not due to a direct toxic effect but to stimulation of a host-mediated response.

Introduction

Many extracts of marine organisms besides venoms and poisons have drug-like effects on mammalian and non-mammalian tissues, including tumors [8]. Most of these extracts are cytotoxic in action and toxic to the host. Chemotherapeutic agents that are cytotoxic toward cancer cells are needed, as are agents that stimulate the host's immunological defenses against neoplastic growth. Such immunopotentiators could combat cancer, reverse immunosuppression, and help in immunodeficiency and chronic infection. Polysaccharides with antitumor activity have been isolated from higher plants, fungi, lichen, bacteria, and yeast [20]. Few attempts have been made to obtain immunopotentiating agents from marine animals. Crude macromolecular fractions from the extracts of some marine animals and a glycoprotein fraction from water extracts of *Patinopecten yessoensis* (scallop) have antitumor activity [14, 16, 17]. This activity is due to stimulation by the fractions of host-mediated responses. In this paper, we studied the separation and properties of a fraction with antitumor effects from *Haliotis discus hannai* (*H. discus Hannai,* abalone).

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Materials and methods

Collection, extraction, and fractionation of abalone. Raw abalone, *H. discus hannai,* was collected from the coastal region of Sanriku, Iwate, Japan, in November 1982. The purification procedure is summarized in Fig. 1. Extract liquor (450 l) obtained from raw abalone (600 kg) by heat-treating at 90 °C for 10 min was provided by a manufacturing factory. The liquor was concentrated by evaporation under reduced pressure at below 40 °C, dialyzed against running water for 48 h, and centrifuged. Ultafiltration of the supernatant was done with an XM-50 membrane (Amicon Corp., Lexington, Mass, USA). The macromolecular fraction with molecular weight greater than 50000 daltons was put on a column of DEAE Sepharose CL-6B equilibrated with 0.01 *M* phosphate buffer (pH 7.4). After being washed with the same buffer, the column was eluted stepwise with 0.4 and then 1.0 *M* NaCl in this buffer. The eluate was collected in three fractions. After concentration, dialysis, and lyophilization, the yields of fractions 1, 2, and 3 were 14.3%, 48.5%, and 32.9%, respectively. Fraction 2, which had the highest tumor inhibition ratio, was put on a column packed with DEAE-Sephacel equilibrated with 0.05 *M* Tris-HCl buffer (pH 8.0). An unadsorbed fraction (fraction 4) was eluted with the starting buffer, and fractions 5 and 6 were eluted with 0.5 and 0.8 *M* NaCl in the same buffer. Fraction 7 wae eluted with 0.1 *M* NaOH in the same buffer. The fractions were dialyzed against distilled water for 48 h and then lyophilized. The yields of fractions 4, 5, 6, and 7 were 8.3%, 30%, 2.1%, and 0.7%, respectively. Fraction 5, which had the highest tumor inhibition ratio, was further purified on the same kind of column with a linear gradient from 0 to 0.5 *M* NaCl in Tris-HCl buffer (pH 8.0). The eluate was collected into four fractions, 8, 9, 10, and 11 (Fig. 2). After dialysis and lyophilization, the yields of the fractions were 4.9%, 38.3%, 25.6%, and 5.0%, respectively.

Chemical analysis of fraction 9. Total carbohydrates were measured by the phenol-sulfuric acid method with D-glucose as the standard [3]. The composition of neutral sugars as dansyl hydrazones after hydrolysis in 4 *N* HCl at 100 °C for 2 h was analyzed using HPLC [2, 10]. Total proteins were assayed by the Lowry method with egg albumin as the standard [9]. The composition of amino acids was identified by a precolumn derivatizing method with dabsyl chloride after the sample had been hydrolyzed with 6 *N* HCl at 110 °C for 24 h [11].

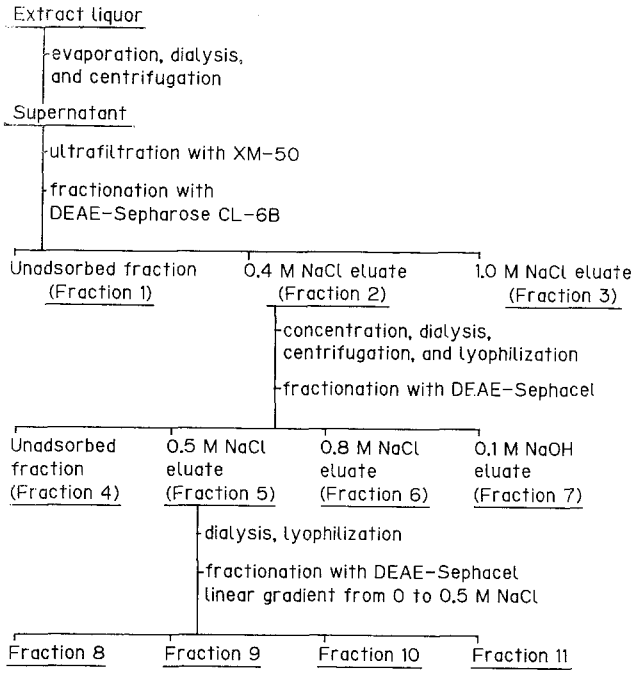

Fig. 1. Preparation of fraction 9 from the aqueous extract of *H. discus hannai*

Mice. Female ICR-JCL mice weighing about 23 g purchased from CLEA Japan, Inc., Tokyo, and female BALB/c and CD-1 nu/nu mice weighing about 20 g purchased from Charles River Japan, Inc., Atsugi were used. BALB/c nu/nu mice were from the Shizuoka Laboratory Animal Center, Shizioka, Japan.

Tumors. Sarcoma 180 and Meth-A fibrosarcoma were initially supplied by the Sloan-Kettering Institute, New York, NY, USA, and they have been maintained in the National Cancer Center Research Institute in ascites form. Unless otherwise stated, 0.05 ml (about 6×10^6 cells) of 7-day-old ascites tumor was transplanted s.c. into the right flank of the mice. Spontaneous regression of these tumors has never been observed.

L5178Y lymphoma cells as the target cells were used in vitro. The cells were maintained in RPMI 1640 medium

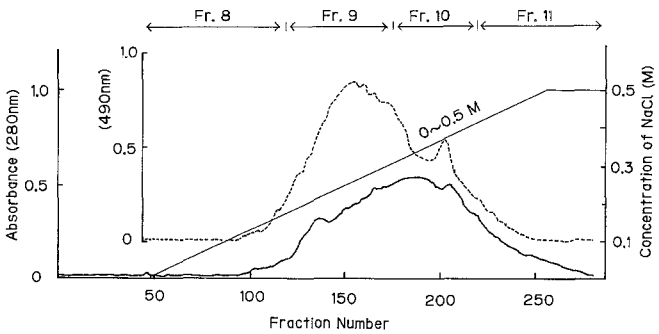

Fig. 2. DEAE-Sephacel column chromatography of fraction 5. Column size was 3.2 cm × 40 cm. The column was eluted with 400 ml of 0.05 *M* Tris-HCl buffer (pH 8.0) and then by a linear gradient of 0–0.5 *M* NaCl in the buffer (8-ml fractions). ———, absorbance at 280 nm; ------, absorbance at 490 nm in phenol-H_2SO_4 method for sugars. Fractions were pooled as indicated by arrows

(Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) with 10% heat-inactivated fetal bovine serum (GIBCO Laboratories, Grand Island, NY, USA) and kanamycin (50 μg/ml); this is referred to as RPMI-FBS.

Assay of antitumor activity. Test samples at suitable concentrations in 0.1 ml of physiological saline were sterilized with an Acrodisc disposable filter assembly (Gelman Sciences, Inc., Ann Arbor, Mich, USA) and injected into the lesion (i.l.) or i.v. on days 5, 7, and 9 after tumor inoculation unless otherwise noted, by which time the tumors had reached a mean diameter of 4 mm. Mice receiving similar treatment with physiological saline were the controls. At the end of the 5th week, the mice were killed and the tumors removed and weighed. Inhibition ratios were calculated by: inhibition ratio (%) = $[(A-B)/A] \times 100$, where A was the mean tumor weight of the control group, and B that of the treated group. Complete regression indicated the ratio of the number of mice with complete tumor regression over the total number of mice tested.

In vivo treatment with carrageenan. Carrageenan (Type V, Sigma Chemical Co., St. Louis, Mo, USA) was suspended in saline by heating at 60 °C for 10 min and injected i.p. at a dose of 80 mg/kg on days 4, 6, and 8 after tumor inoculation.

In vitro cytocidal test. Ascites tumor cells (10^5 cells) of sarcoma 180 or Meth-A fibrosarcoma in 0.5 ml of RPMI-FBS containing various concentrations (20–500 μg/ml) of fraction 9 or its autoclaved product were incubated at 37 °C for 48 h in moist air containing 5% CO_2. The viability of these cells incubated with or without the samples, assessed by exclusion of trypan blue, was over 95%. The cells were counted under a phase-contrast microscope. Inhibition ratios were calculated by: inhibition ratio (%) = $[(A-B)/A] \times 100$, where A was the mean number of cells incubated without the samples, and B that of cells incubated with the samples.

Preparation of effector cells. Peritoneal macrophages were isolated by the method of Kumagai et al. [7]. The cells were harvested by washing with 5 ml of RPMI 1640 medium containing sodium heparin (2 units/ml) and kanamycin (50 μg/ml). Then they were washed twice with RPMI 1640 medium containing kanamycin (50 μg/ml) and suspended in RPMI-FBS. To separate the adherent cells, a supension of the cells was put into a plastic dish (No. 3003, Becton Dickinson Labware, Oxnard, Calif., USA) that had been coated overnight with heat-inactivated fetal bovine serum, and incubated at 37 °C in moist air containing 5% CO_2 to allow the macrophages to become attached to the dish. After 20 min, the nonadherent cells were decanted, and the dish was washed 5 times with cold RPMI-FBS. The remaining cells were then removed from the dish by incubation at 4 °C for 40 min in phosphate-buffered saline (pH 7.2) containing 0.2% EDTA and 5% heat-inactivated fetal bovine serum.

Alveolar macrophages were isolated by the method of Howard et al. [5]. Lungs were cut into small pieces with scissors, vigorously pipetted, and then squeezed through fine gauze with RPMI 1640 medium containing sodium heparin (2 units/ml) and kanamycin (50 μg/ml). Erythrocytes were lysed by osmotic shock. After washing, the lung

cells were suspended in RPMI-FBS, and the nucleated cells counted. Alveolar macrophages were then separated from the lung cells by the method described above.

Assay of in vitro cytostatic activity of peritoneal and alveolar macrophages. To assay cytostatic activity, peritoneal and alveolar macrophages (effector cells) were suspended at a concentration of 10^6 cells/ml in RPMI-FBS. A mixture of 0.1 ml of effector cells and 0.1 ml of L5178Y cells (10^5 cells/ml) was introduced into wells of a tissue culture plate (No. 25860, Corning Glass Works, Corning, NY, USA) and incubated at 37 °C for 48 h in moist air containing 5% CO_2. The cultures were pulsed with 0.4 μCi of ^{3}H-thymidine (sp. act., 84.0 Ci/ml, New England Nuclear Corp., Boston, Mass., USA) per well for 4 h before incubation was stopped. Then, the lymphoma cells were harvested using a cell harvester, and the radioactivity in the acid-insoluble fraction of the cells was counted in a liquid scintillation counter (Model LS-9000, Beckman Instruments, Inc., Irvine, Calif., USA). The percentage of cytostatic activity was calculated by; cytostatic activity (%) = $(1\text{-A/B}) \times 100$, where A was the mean radioactivity incorporated into a L5178Y cell culture after incubation with effector cells from treated mice, and B was the mean radioactivity after incubation with effector cells from control mice. There was no significant difference between L5178Y cells incubated with and without normal effector cells.

Statistical analysis. We used Student's *t*-test to study statistical significance. All *P* values below 0.05 were taken to be significant.

Results and discussion

Of all fractions obtained, fraction 9 had the strongest inhibitory activity against sarcoma 180 cells transplanted into ICR mice when given i.l. at a dose of 80 mg/kg three times (Table 1). The fraction was not completely purified, but its major component was glycoprotein with 22% of total carbohydrate as glucose and 44% of total protein as egg albumin. Sugar analysis gave D-galactose as a major component. D-Ribose, D-xylose, D-mannose, L-fucose, and D-glucose were also detected, but their amounts were less than one-third that of D-galactose. The fraction also contained large amounts of tyrosine, asparagine, glycine, and threonine. These totalled nearly 50% of the amino acid residues. Only small amounts of half-cystine, histidine, methionine, and lysine were detected. This fraction had a molecular weight of about 290000 daltons as estimated by the elution pattern of Sephacryl S-300 gel filtration, and elementary analysis (%) showed C, 40.6; H, 6.6; N, 7.6; and ash, 5.0.

One question about the antitumor action of fraction 9 is whether its systemic injection is effective. To answer this, we examined the effect of the fraction on sarcoma 180 solid tumors in ICR mice by i.v. injection (Table 2). Fraction 9 gave maximum tumor suppression at a dose of 80 mg/kg, with an inhibition ratio of 75.8%, when injected i.v. three times. This fraction also gave a high inhibition ratio, 79.1%, against sarcoma 180 transplanted i.d. into ICR mice, with complete disappearance of the tumor in 3 out of 6 mice when injected i.v. at a dose of 80 mg/kg. Fraction 9 was heat-labile because its autoclaved product had less antitumor activity (37.5%) when given i.v. at a dose of 80 mg/kg on the same schedule. This fraction also had inhibition ratios over 75% when injected i.v. at a dose of 100 mg/kg three times on all schedules tested except for injections on days 1, 3, and 5 after tumor inoculation (Table 3). Thus, the antitumor activity had optimum dosage and timing.

The antitumor effects of fraction 9 in other tumor-mouse systems were tested (Table 4). This fraction gave an inhibition ratio of 100% with complete tumor regression

Table 1. Effects of fractions from an aqueous extract of *H. discus hannai* on sarcoma 180 solid tumors in ICR mice by i.l. injection

Fraction no	Dose (mg/kg × 3 days)	Mean tumor weight (g)	Tumor inhibition ratio (%)	Complete regression[a]
1	400	4.0 ± 1.7	38.5	0/6
2	400	0.4 ± 0.5[b]	93.8	3/6
	200	2.4 ± 1.4	63.1	0/6
3	400	4.7 ± 1.9	27.7	0/6
	200	5.3 ± 1.4	18.5	0/6
Control		6.5 ± 2.0		0/6
4	200	1.1 ± 2.5[b]	82.8	2/6
	80	4.7 ± 1.6	26.5	0/5
5	200	0.5 ± 0.8[b]	92.1	3/6
	80	3.0 ± 2.1[c]	53.1	1/6
6	200	3.2 ± 1.3[b]	50.0	0/6
	80	5.0 ± 2.3	21.8	0/6
7	200	1.1 ± 2.7[b]	83.0	3/6
Control		6.4 ± 2.0		0/6
8	80	1.0 ± 1.2[b]	84.8	1/6
9	80	0.2 ± 0.3[b]	96.9	5/6
10	80	2.4 ± 0.2[c]	63.6	0/6
11	80	2.6 ± 3.4	60.6	0/6
Control		6.6 ± 3.8		0/6

[a] Number of tumor-free mice/number of mice tested
[b] $P < 0.01$ compared with control
[c] $P < 0.05$ compared with control

against Meth-A fibrosarcoma transplanted s.c. into BALB/c mice when given i.l. at a dose of 200 mg/kg three times on days 5, 7, and 9 after tumor inoculation. The ratio was 66.7% with complete disappearance of the tumor in 1 out of 6 mice when given i.v. on the same schedule. This fraction had antitumor activity in both allogeneic and syngeneic tumor systems when given locally or systemically.

The biological properties of fraction 9 and of the glycoprotein fraction (fraction DC-1) from *Patinopecten yessoensis* [17] were similar. Fraction 9 lost its activity in vivo after being autoclaved, but fraction DC-1 did not. Fraction 9 significantly inhibited the growth of Meth-A solid tumors in BALB/c mice even by i.v. injection, whereas fraction DC-1 did not. These were the main differences between fraction 9 from abalone and fraction DC-1 from scallop.

Another question about the antitumor effects of fraction 9 is whether its activity is due do direct cytotoxicity or is mediated through the cellular immune response of the host. Fraction 9 was slightly cytostatic to sarcoma 180 cells and Meth-A fibrosarcoma cells in vitro, with inhibition ratios of 34.6% and 28.4%, respectively, at a dose of 500 μg/ml. Autoclaving this fraction decreased its antitumor activity against sarcoma 180 solid tumors in ICR mice, but increased its direct effects in vitro on the growth of sarcoma 180 cells; the autoclaved product gave an inhibition ratio of 50.6% at a dose of 500 μg/ml. Fraction 9 had an optimum dosage and timing, and characteristics of biological response modifiers such as lentinan [4]. The possibility of direct toxic effects of fraction 9 on tumors in vivo could not be ruled out, but its antitumor action seemed to be mediated through the cellular immune response of the host. The effects when this fraction was given i.l. on sarcoma 180 solid tumor in CD-1 nu/nu mice and on Meth-A fibrosarcoma solid tumor in BALB/c nu/nu mice were much less than in ICR or BALB/c mice (Table 4). This result indicates that T cells are important in the antitumor activity of fraction 9.

Macrophages are effector cells essential to the host's defense against tumor growth [1, 6, 13, 15, 19]. If macrophages are involved in the antitumor activity of fraction 9, an agent such as carrageenan, which decreases macrophage numbers and function, should reduce the effect of this fraction. In fact, suppression of macrophage function in vivo by i.p. injections of carrageenan significantly decreased the antitumor effect of fraction 9 (Table 5). In ad-

Table 2. Effects of fractions from an aqueous extract of *H. discus hannai* on sarcoma 180 solid tumors in ICR mice by i.v. injection

Fraction no	Dose (mg/kg × 3 days)	Mean tumor weight (g) (treated/control)	Tumor inhibition ratio (%)	Complete regression[a]
4[b]	40	2.3 ± 1.8/7.0 ± 2.0[c]	67.1	0/6
	120	4.6 ± 4.4/6.4 ± 3.8	28.1	0/6
5[b]	240	2.0 ± 1.8/7.0 ± 2.0[c]	71.4	2/6
	120	4.6 ± 4.0/8.6 ± 3.6	46.5	0/6
	40	9.0 ± 3.2/8.6 ± 3.6	–4.6	0/7
9[b]	40	4.5 ± 3.7/6.2 ± 2.5	27.4	0/6
	80	1.5 ± 1.1/6.2 ± 2.5[c]	75.8	0/6
	160	1.9 ± 0.3/6.2 ± 2.5[c]	69.4	0/6
	320	3.6 ± 2.1/6.2 ± 2.5	41.9	0/6
10[b]	80	4.4 ± 3.4/8.6 ± 3.6	33.3	0/6
9[d]	80	1.5 ± 2.4/7.2 ± 5.1[e]	79.1	3/6
	8	4.5 ± 2.4/7.2 ± 5.1	37.5	0/6
Autoclaved 9[d]	80	4.5 ± 2.1/7.2 ± 5.1	37.5	0/6

[a] Number of tumor-free mice/number of mice tested
[b] Sarcoma 180 cells were inoculated s.c. into the right flank of ICR mice and the test samples were injected i.v.
[c] $P < 0.01$ compared with control
[d] Sarcoma 180 cells were inoculated i.d. into the back of ICR mice and the test samples were injected i.v.
[e] $P < 0.05$ compared with control

Table 3. Effects of timing of i.v. administration of fraction 9 on sarcoma 180 solid tumors in ICR mice[a]

Dose (mg/kg × 3 days)	Days of injections	Mean tumor weight (g)	Tumor inhibition ratio (%)	Complete regression[b]
100	1, 3, 5	3.3 ± 1.6[c]	46.8	0/5
	3, 5, 7	0.9 ± 0.8[d]	85.5	0/6
	5, 7, 9	1.0 ± 0.6[d]	83.9	0/6
	7, 9, 12	1.5 ± 1.2[d]	75.8	0/6
	9, 12, 15	1.2 ± 1.4[d]	80.6	0/6
Control		6.2 ± 2.4		0/6

[a] Sarcoma 180 cells (6×10^6) were inoculated s.c. on day zero
[b] Number of tumor-free mice/number of mice tested
[c] $P < 0.05$ compared with control
[d] $P < 0.01$ compared with control

dition, the peritoneal and alveolar macrophages from mice given fraction 9 decreased the incorporation of ^{3}H-thymidine into L5178Y cells in vitro, whereas macrophages from normal mice did not. Fraction 9 activated the cytostatic activity of both peritoneal and alveolar macrophages in vivo; such activity was maximum on days 3–5 and on days 1–3 for peritoneal and alveolar macrophages, respectively (Table 6). We confirmed by use of a phase-contrast microscope that both kinds of macrophages from mice given this fraction 3 days earlier decreased the proliferation of L5178Y cells in vitro. Fraction 9 was not contaminated with endotoxin; a *Limulus* lysate assay [12] was negative (data not shown). These results show that macrophages participated in the antitumor activity of fraction 9. We concluded that its antitumor activity is mediated by the cellular immune response of the host in which both T cells and macrophages are important.

We compared the antitumor effect of fraction 9 with that of lentinan, (1→3)-β-D-glucan, a biological response modifier. The effects of antitumor polysaccharides differ among mouse strains, BALB/c mice are especially weak responders to lentinan [18]. In contrast, fraction 9 had an antitumor effect on Meth-A fibrosarcoma in BALB/c mice. Fraction 9 and lentinan have a different antitumor spectrum, at least in part, although the antitumor activity of both agents is mediated by the cellular immune response of the host in which both T cells and macrophages are important.

Table 4. Effects of fraction 9 on other tumor systems at a dose of 200 mg/kg for 3 days

Tumor	Strain of mouse	Administration	Mean tumor weight (g) (treated/control mice)	Tumor inhibition ratio (%)	Complete regression[a]
Meth-A fibrosarcoma	BALB/c	i.l.	0 /2.4±1.5[b]	100	7/7
Meth-A fibrosarcoma	BALB/c	i.v.	0.8±1.5/2.4±1.5[c]	66.7	1/6
Meth-A fibrosarcoma	BALB/c nu/nu	i.l.	2.7±1.0/4.3±1.8	37.2	0/6
Sarcoma 180	CD-l nu/nu	i.l.	2.7±2.0/3.8±1.1	28.9	0/6

[a] Number of tumor-free mice/number of mice tested
[b] $P < 0.01$ compared with control
[c] $P < 0.05$ compared with control

Table 5. Effects of carrageenan on the antitumor activity of fraction 9[a]

Treatment	Administration	Mean tumor weight (g ± SD)	Tumor inhibition ratio (%)	Complete regression[a]
None		6.3±3.6		0/16
Fraction 9	i.l.	0.3±0.6[c]	95.2	6/9
Fraction 9 plus carrageenan	i.l. i.p.	3.3±1.8	47.6	0/5
Fraction 9	i.v.	2.1±2.7[d]	66.6	1/6
Fraction 9 plus carrageenan	i.v. i.p.	6.6±3.4	−4.7	0/6
Carrageenan	i.p.	8.4±1.8	−33.3	0/6

[a] Sarcoma 180 tumor cells (2×10^6) were inoculated s.c. on day zero. Fraction 9 (100 mg/kg) dissolved in 0.1 ml of physiological saline was injected i.v. or i.l. on days 5, 7, and 9. Carrageenan (80 mg/kg) dissolved in 0.2 ml of physiological saline was injected i.p. on days 4, 6, and 8
[b] Number of tumor-free mice/number of mice tested
[c] $P < 0.01$ compared with control
[d] $P < 0.05$ compared with control

Table 6. In vivo activation of cytostatic activity of fraction 9 mediated by peritoneal and alveolar macrophages

Effector cells[a]	Cytostatic activity (%)[b]				
	Day 1	Day 3	Day 5	Day 8	Day 12
Peritoneal macrophages	34.7[c]	79.8[c]	85.5[c]	21.9[c]	−0.5
Alveolar macrophages	50.6[c]	41.7[c]	0.4	3.0	N.T.[d]

[a] Macrophages were harvested on the day indicated after a single i.v. injection of fraction 9 at a dose of 100 mg/kg
[b] The ability of effector cells from the treated mice to inhibit incorporation of ^{3}H-thymidine into L5178Y cells in vitro at an effector: target cell ratio of 10:1 was compared to that of effector cells from control mice
[c] $P < 0.001$ compared with controls
[d] Not tested

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