Pharmacological studies on a plant lectin, Aloctin A. I. Growth inhibition of mouse methylcholanthrene-induced fibrosarcoma (Meth A) in ascites form by Aloctin A[1]

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Summary. A glycoprotein isolated from *Aloe arborescens* Mill markedly inhibited the growth of a syngeneic transplantable fibrosarcoma of mice, Meth A, in ascites form. There is evidence that the inhibition mechanism is host-mediated and not a direct toxic effect on the tumor cell.

A variety of immunostimulators have been reported as antitumor substances, such as levamisole[4], BCG[5], OK-432 (streptococcal preparation)[6], lentinan[7], PS-K (protein-bound polysaccharide isolated from *Coriolus versicolor*)[8] etc. As reported previously[9], in our laboratory 2 lectins, P-2 and S-1 fractions were isolated from leaves of *Aloe arborescens* Mill and were designated Aloctin A and Aloctin B, respectively. Aloctin A (Alo A) is a glycoprotein, is easily water-soluble, has a mol wt of 18,000, consists of 2 subunits, α and β, and exhibits various biological activities, such as hemagglutination, mitogenic activity for lymphocytes, binding reactivity for serum proteins and complement 3rd component (C3) activation via the alternative pathway[9,10]. In this paper we demonstrate that Alo A inhibits the growth of methylcholanthrene-induced fibrosarcoma (Meth A) in syngeneic BALB/c mice and that Alo A was not directly cytotoxic to tumor cells in vitro.

BALB/c mice, 5-6 weeks old, were used and Meth A was maintained in ascites form by weekly i.p. passage in BALB/c mice. Washed 1×10^6 7-day-old Meth A cells in 0.1 ml of Eagle's MEM were injected into the peritoneal cavity. The test samples were administeres i.p. at an appropriate concentration in saline, once daily for 5 days, starting 24 h after tumor implantation. Antitumor activity was evaluated by the total packed cell volume ratio (T/C %) calculated from collected whole ascites obtained from mice anesthetized with ether. A representative experiment is shown in table 1. Alo A obviously inhibited the growth of Meth A and administration at a dose of 10 mg/kg/day, for 5 days, remarkably inhibited it ($p < 0.001$). No clear dose-response was observed within the experiment. OK-432 which is used as a clinical injection in immunotherapy of cancer patients, used as a positive control, also inhibited the growth of Meth A ($p < 0.005$). OK-432 was kindly provided by Chugai Pharmaceutical Co., Tokyo. The antitumor activity of Alo A is obvious and seems to be higher than that of OK-432. It was important to determine whether this activity was due to cytotoxicity of Alo A for tumor cells or host-mediated effects of Alo A, since Alo A was administered i.p. Therefore, the effect of Alo A on the growth in vitro of Meth A and the other cell lines was examined by ^{3}H-thymidine uptake. RPMI 1640 medium (Gibco Laboratories, New York, USA) supplemented with 10% fetal calf serum (Microbiological Associates, Maryland, USA), 2 mM L-glutamine, 3% sodium bicarbonate, 100 IU/ml penicillin, and 100 µg/ml streptomycin, was used as tissue culture medium. Various concentrations of Alo A in 100 µl were added to 100 µl of cell suspension, each containing $2.5\text{-}5 \times 10^3$ cells and the mixture was incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C for 28 h. After 24 h of incubation, 1 µCi/well of ^{3}H-thymidine (The Radiochemical Centre, Amersham, England; sp. act. 5 Ci/mmole) was added. After a further 4 h of incubation, cells were harvested on glass-fiber filters (Reeve Angel Co., New Jersey, USA) by the use of a Multiple Automatic Samole Harvester MASH II (Microbiological Associates, Maryland, USA). The dried filters were put into a scintillation cocktail of 5 g PPO and 0.1 g POPOP/l of toluene and counted in a Beckman LS-230 Liquid Scintillation System (Beckman Instruments, California, USA). Alo A had almost no inhibitory effect on the growth of tumor cell lines tested involving Meth A up to a concentration of 200 µg/ml (table 2). Lower concentrations of Alo A rather stimulated the growth of some tumor lines, but the reason for this is not known. This result suggests that Alo A is not directly cytotoxic to tumor cells.

Although the antitumor activity of some lectins has been examined by others, only moderate effects were reported for concanavalin A[11,12] and the highly toxic lectins, ricin and abrin[12,14]. Alo A had a marked antitumor activity and was not directly cytotoxic. The mechanism of this activity is obscure, but is considered to be host-mediated. Since Alo A agglutinates tumor cells[15], it could be considered that tumor immunogenicity was improved by being bound with Alo A as Martin et al.[16] showed with Con A. As described above, Alo A activates complement 3rd component (C3) via the

Table 1. The anti-tumor activity of Aloctin A against sarcoma Meth A (ascites form) in BALB/c mice

Treatment	Dose (mg/kg/day × days)	Average TPCV[a] (ml)	T/C ratio (%)	Complete inhibition
None		0.61		0/10
Aloctin A	10 × 5	0.05	7.7**	4/6
Aloctin A	2 × 5	0.37	60.4	1/6
Aloctin A	0.4 × 5	0.41	66.7	1/6
OK-732[b]	2.5 KE/head × 5	0.18	30.3*	3/6

Antitumor test: 5-week-old BALB/c mice were used for this test. The tumor used was methylcholanthrene-induced fibrosarcoma (Meth A) maintained in the ascites form. 1×10^6 washed cells of Meth A were implanted i.p. into the mouse. Aloctin A as injected i.p. once daily for 5 days, starting 24 h after tumor implantation. Antitumor activity was evaluated by the total packed cell volume ratio (T/C %) on the 7th day.

[a]Total packed cell volume, [b]Streptococcal preparation (Chugai Pharmaceutical Co., Tokyo). * $p < 0.005$, ** $p < 0.001$, Significantly different from control.

Table 2. Cytotoxicity of Aloctin A in vitro

Concentration of Aloctin A (µg/ml)	^{3}H-TdR incorporation (cpm)[a] Meth A	EL 4	P815	BW5147	YAC
None	42890	68351	39865	14989	7120
0.02	43195	87497	67506	24247	8345
0.2	40512	90111	74904	24468	7648
2.0	39250	87798	68245	28597	7618
20.0	45924	80864	63572	19300	6835
200.0	45537	66979	49111	11250	818

Cytotoxicity test: Various concentrations of Aloctin A in 100 µl of cell suspension, each containing $2.5\text{-}5 \times 10^3$ cells. The mixture was incubated at 37 °C for 28 h in a humidified atmosphere of 5% CO_2 and 95% air. After 24 h, 1 µCi/well of ^{3}H-thymidine was added. After a further 4 h of incubation, radioactivity incorporated into DNA was determined.

[a]Mean cpm of 3 wells.

alternative pathway and binds to some serum proteins such as α_2-macroglobulin and α_1-antitrypsin[10]. It could be concerned with the immune reaction of the hosts. Further immunological studies are under way.

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Metallothionein induced in the earthworm[1]

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Summary. One of the 3 different molecular weight cadmium-binding proteins induced in the earthworm was characterized as a metallothionein; this characterization was based on a high cysteine and cadmium content, low molecular weight, heat-stability, and mercaptide bonding.

The earthworm, *Eisenia foetida*, shows a marked tolerance to cadmium and accumulates the metal to a degree related to its concentration in composted sewage sludges[4]. The accumulated cadmium was mostly found in the cytosol fraction; this is known to be usual in animals, including mammals[5]. The distribution profile of cadmium among the soluble proteins, however, was quite different from that in mammals; cadmium was not only found in the metallothionein fraction (apparent mol. wt of about 10,000), but also in higher and lower mol. wt fractions (estimated mol. sizes of 63,000–70,000 and less than 2000, respectively)[6]. A lower mol. wt cadmium-binding protein than the mammalian metallothionein has been isolated from a fungus, *Neurospora crassa*, and characterized as a metallothionein with a mol. wt of 2000[7]. This is the first observation of the induction of cadmium-binding proteins with 3 different mol. wts. The present communication reports some characterizations of the earthworm cadmium-binding proteins and defines one of the cadmium-binding proteins as a metallothionein from its amino acid composition, metal content, and other features accepted as being characteristic of metallothioneins[5].

The earthworm, *Eisenia foetida*, was grown for 60 days in a composted sewage sludge which contained 400 µg Cd/g dry wt. The earthworms were homogenized after discharging excreta, and heat-unstable proteins were removed from the homogenate by heating at 80 °C. Typical gel filtration chromatograms of the heat-denatured supernatant on Sephadex G-75 and SW 3000 columns are shown in figure 1. Most of the zinc distributed in the high mol. wt fraction was removed by heat-treatment without affecting the distribution profile of cadmium. The highest mol. wt cadmium-binding protein (EW-I) on a Sephadex G-75 column was eluted as a broad peak at a retention time of 17.2 min from an SW cokumn. On the other hand, the other 2 cadmium-binding proteins separated on a Sephadex G-75 column, EW-II and -III, were separated into several peaks on an SW column due to the cation exchange chromatographic property of the column with elution at alkaline pH[8].

Further separation of the 3 cadmium-binding proteins (EW-I, -II, and -III) was carried out on a DEAE Sephadex A-25 column, one of the most usual procedure for separation of metallothionein into isometallothioneins. EW-I could not be eluted from the column using conditions the same as, or more drastic than those used for the separation

Amino acid composition, total sugar and metal contents of earthworm cadmium-binding protein-IIB (EW-IIB)[a]

Amino acid composition	Mole %	Residues[b] / Molecule
Half-cystine	15.4	14
Aspartate	9.8	9
Threonine	5.7	5
Serine	6.1	6
Glutamate	8.5	8
Glycine	17.0	15
Alanine	10.0	9
Valine	4.6	4
Methionine	-	-
Isoleucine	2.2	2
Leucine	5.1	5
Tyrosine	-	-
Phenylalanine	Trace	Trace
Lysine	8.6	8
Histidine	-	-
Arginine	3.0	3
Proline	4.0	4
Metal content (mole %): Cd 93.9, Zn 2.8, Cu 3.3		
SH/metal ratio: 2.4		
Total sugar: -		

[a] EW-IIB was hydrolyzed in 6 N HCl at 110 °C for 20 h. Half-cystine and methionine were analyzed after performic acid oxidation. Values are expressed as percent of the total number of residues. Total sugar and metal contents were determined by the H_2SO_4-phenol assay[9] and atomic absorption spectrophotometry, respectively.
[b] Isoleucine was assumed to be 2 residues per molecule.