Isolation of Galactosaminoglycan Moiety (CO-N) from Protein-Bound Polysaccharide of *Cordyceps ophioglossoides* and Its Effects against Murine Tumors

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A galactosaminoglycan moiety was obtained from an antitumor polysaccharide fraction (SN-C) isolated from *Cordyceps ophioglossoides* culture. SN-C was subjected to sonication, then a protein-bound galactosaminoglycan (CO-N) was isolated specifically by precipitation with 10% ammonium hydroxide.

When given intraperitoneally to mice, CO-N inhibited the proliferation of sarcoma 180 cells inoculated into the peritoneal cavity and exhibited a marked life-prolonging effect against ascitic tumors such as Ehrlich carcinoma and IMC carcinoma. CO-N also showed an inhibitory effect against solid Ehrlich carcinoma when given intratumorally and significantly inhibited the growth of a syngeneic solid tumor (MM46 mammary carcinoma) upon intravenous administration at a low dose.

CO-N showed a cytocidal effect against cultured cells of IMC and P388D$_1$ *in vitro*. Flow cytometric analysis demonstrated that fluorescein isothiocyanate–CO-N binds to the surface of Ehrlich cells.

Keywords *Cordyceps ophioglossoides*; SN-C (polysaccharide fraction); galactosaminoglycan; CO-N (protein-bound galactosaminoglycan); antitumor activity; cytocidal effect; fluorescein isothiocyanate–CO-N

Introduction

Among a large number of antitumor polysaccharides derived from microorganisms,[1] those having aminosugars as a main constituent are very few. Recently, chitin, chitosan and chitooligosaccharides have been reported to have antitumor activities.[2]

In our previous reports, it was shown that a polysaccharide fraction (SN-C) obtained from the culture of *Cordyceps ophioglossoides* exerts both host-mediated and direct antitumor activities,[3] and the host-mediated effect was ascribed to β(1—3)-glucan (CO-1) of SN-C.[4]

In the present study, we isolated another constituent, a protein-bound galactosaminoglycan (CO-N), from SN-C solution by the removal of β(1—3)-glucan after sonication. A direct antitumor activity of CO-N was observed.

Materials and Methods

Isolation of Galactosaminoglycans *C. ophioglossoides* culture was centrifuged to remove mycelia. Charcoal was added to the supernatant solution, which was then filtered, and an equal volume of ethanol was added to the filtrate. The resulting pricipitate was collected, and SN-C was obtained by drying *in vacuo*. SN-C was resuspended in 0.01 M acetic acid to a concentration of 0.5%, sonicated at 4 °C, and then centrifuged. The supernatant solution was incubated at 60 °C for 30 min and then centrifuged to remove a white precipitate (CO-1), and 10% aqueous ammonium hydroxide was added to make pH 6.0—9.0. The resulting precipitates were redissolved in 0.01 M acetic acid and reprecipitated with ammonium hydroxide. Those precipitates were washed with water to give purified galactosaminoglycan. The finally obtained fraction of galactosaminoglycan (CO-N), was eluted as a single peak in gel filtration on Toyo Pearl HW-55 (fine).

Analytical Methods Hexose content was measured by the phenol–sulfuric acid method,[5] and aminosugar content by the indole–hydrochloric acid method.[6] 2-Amino-2-deoxy-D-galactose was determined by using an amino acid analyzer (Hitachi 835; Hitachi Ltd., Tokyo, Japan) after acid hydrolysis (4 N HCl 100 °C, 22 h) and protein content by the Lowry–Folin method.[7]

Preparation of the Sample CO-N was dissolved in 0.01 N acetic acid, neutralized with sodium hydroxide, and then equilibrated with glucose. After being sterilized by passage through a 0.45 μm Mirex filter (Millipore Co., Bedford, MA., U.S.A.), the solution was diluted with 0.01 N sodium acetate–glucose solution and used for the experiments.

Experimental Animals Female ICR mice (7 weeks of age, 20—25 g) were purchased from Clea Japan Inc., Tokyo. Female C3H/He mice (7 weeks of age, 20—25 g) were purchased from Shizuoka Laboratory Animal Center (SLC, Hamamatsu, Japan). Female DBA/2 and CDF$_1$ mice (7 weeks of age, 20—25 g) were purchased from Charles River Japan, Inc., Atsugi.

Tumors Sarcoma 180, Ehrlich carcinoma and MM46 mammary carcinoma were kindly provided by the National Cancer Center; L1210 leukemia and P388 leukemia were kindly provided by the National Defense Medical College; and IMC carcinoma by Kitasato University. An ATCC strain of P388D$_1$ lymphoid neoplasm cell was purchased from Dainippon Pharmaceutical Co., Ltd., Osaka, Japan. Each tumor was maintained in the peritoneal cavity of inbred mice by serial passages.

Antitumor Experiments 1) Inhibitory Effect on the Proliferation of Intraperitoneally Transplanted Cells: According to the TPCV method of Hoshi *et al.*,[8] 1×10^6 sarcoma 180 cells were inoculated into the peritoneal cavity of ICR mice, and a single dose of CO-N was administered intraperitoneally 24 h after the transplantation. On the 6th day, the cells in the peritoneal cavity were collected with Hanks' balanced salt solution and the packed cell volume was determined and compared with that of the vehicle-control group. The inhibition ratio was calculated by applying the following formula: inhibition ratio (%) $= (1 - T_v/C_v) \times 100$, where T_v is the packed tumor cell volume of the CO-N-treated group and C_v is that of the vehicle-control group.

2) Life-Prolonging Effect on Ascitic Tumors: Animals, tumors, and the number of transplanted cells used in the study were as follows; ICR mice (Ehrlich, 1×10^6), DBA/2 (L1210, and P388, 1×10^5) and CDF$_1$ (IMC, 1×10^5). Various doses of CO-N (1—50 mg/kg) were administered intraperitoneally for 10 consecutive days from the day after tumor transplantation. The mortality of mice was observed for 60 d after the tumor transplantation. The increase in life span (ILS) was calculated according to the following formula: ILS (%) $= (T/C - 1) \times 100$, where T is the median survival time (MST) of the CO-N-treated group and C is that of the vehicle-control group.

3) Effect against Solid Tumors: Ehrlich carcinoma (1×10^6) was inoculated subcutaneously into the right inguinal region of ICR mice. Starting 24 h after the transplantation, CO-N was administered at doses of 0.5—100 mg/kg for 10 consecutive days by the intraperitoneal, intravenous, intramuscular or intratumoral route. MM46 carcinoma was transplanted subcutaneously into the right inguinal region of C3H/He mice. On the 6th day, when the tumor growth was confirmed, CO-N was administered at doses of 1 or 10 μg/kg intravenously or intraperitoneally on days 6, 8, 10, 12 and 14. The time course of tumor growth was determined and the tumor was excised 30 d after the transplantation. The

weight of tumor was compared with that of the control group. The tumor growth inhibition ratio was calculated according to the following formula: inhibition ratio (%) $=(1-T/C)\times 100$, where T is the average tumor weight of the CO-N-treated group and C is that of the vehicle-control group.

4) The Cytocidal Effect *in Vitro*: IMC cells and P388D$_1$ cells were adjusted to concentration of 2×10^5/ml and 1×10^5/ml, respectively, with completely defined serum-free medium HL-1™ (Ventres Laboratories, Inc., U.S.A.). To IMC cells, CO-N was added to give concentrations of 12.5—50 μg/ml at the beginning of culture. The cells were cultured in a CO_2 incubator at 37 °C for 12, 24, 48, or 72 h. To P388D$_1$ cells, CO-N was added to give concentrations of 5—60 μg/ml on the 4th day of culture, and the cells were cultured for a further 12, 24 or 48 h. The number of viable cells after culture was counted by the trypan blue dye exclusion method to evaluate the cytocidal effect.

5) The Analysis of Cell-Bound CO-N by Flow Cytometry: Fluorescein isothiocyanate (FITC)-conjugated CO-N was prepared according to the method of Kawaoi.[9] with minor modifications. Since CO-N was insoluble in an alkaline solution, CO-N was first dissolved in 0.01 N acetic acid and the pH was adjusted to 6.5 with 0.01 N NaOH. FITC solution was added and the crude reactant was purified on Sephadex G-25. The purified FITC–CO-N was allowed to react with phosphate buffered saline (PBS)-washed viable cells of Ehrlich tumors at 4 °C for 30 min. Unbound FITC–CO-N was removed by washing with PBS and the cells were examined under a fluorescence microscope. Some of the cells were analyzed for fluorescence intensity by the use of a FACS analyzer (Beckton Dickinson, CA., U.S.A.).

Results

1) Isolation of CO-N The glucan moiety (CO-1) was removed from SN-C by ultrasonication and heating as described, and CO-1-free solution was used as the starting material. After treatment at various pHs, the yield of precipitate and the content of hexosamine were determined. The yield of precipitate increased as the pH was increased, but the yield remained unchanged at pH higher than 8 (Table I). The precipitate was revealed to be mainly composed of galactosamine by the indole–hydrochloric acid method[6] and by using an amino acid analyzer. The highest recovery of galactosamine was seen when CO-1-free solution was treated at pH 9.0. The precipitate of galactosaminoglycan obtained at pH 9.0 (CO-N) was dissolved in 0.01 N acetic acid and the pH was adjusted to 6.5 with 0.01 N NaOH; the tonicity was adjusted with glucose to give an isotonic solution, and the sample was used for the experiments. The isolation method of CO-N is summarized in Fig. 1.

2) Antitumor Effects a) Effect of CO-N against Intraperitoneally Transplanted Tumor: The inhibitory effect against sarcoma 180 is shown in Table II. It was clear that even at a single dose of 0.25 mg/kg, CO-N exhibited over 95% inhibition of sarcoma 180 growth.

TABLE I. Effect of pH on the Separation of CO-N

pH	Yield[a] (mg)	Component (%)		
		Hexose[b]	Hexosamine[c]	Protein[d]
6.0	0	—	—	—
7.0	12.5	7.3	77.4	5.2
8.0	80.3	5.4	78.3	5.3
9.0	80.6	5.1	80.5	5.6
SN-C	—	75.2	17.3	5.5

a) SN-C (500 mg) was dissolved in 0.01 N acetic acid (100 ml), and heated at 60 °C for 30 min after ultrasonication. *b*) Hexose content was measured by the phenol–sulfuric acid method. *c*) Hexosamine content was measured by the indole–hydrochloric acid method. *d*) Protein content was measured by the Lowry–Folin method.

b) Life-Prolonging Effect of CO-N on Mice Inoculated with Ascitic Tumors: CO-N was administered in various protocols involving the same total dose after tumor

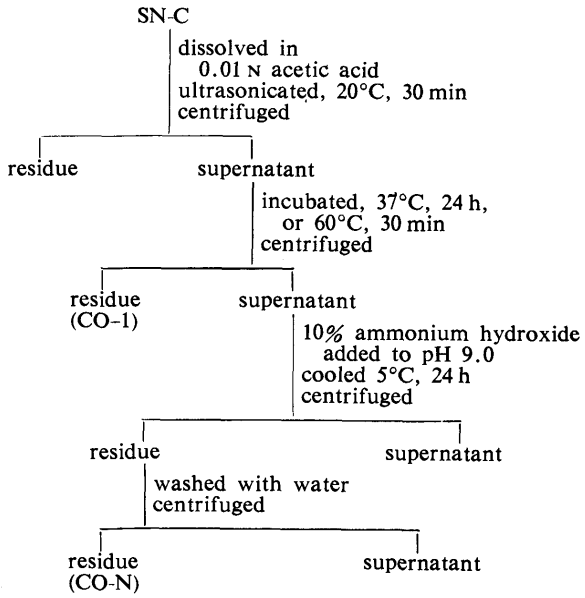

Fig. 1. Purification Procedure of CO-1 and CO-N

TABLE II. Effect of CO-N against Implanted Sarcoma 180 Cells

Dose[a] (mg/kg)	Cell volume[b] (cm^3)	Inhibition ratio (%)
0.25	0.06	96.1
0.5	0.02	98.7
1.0	0.02	98.7
2.0	0.02	98.7
4.0	0.01	99.4
Control	1.55	—

a) The indicated dose of CO-N was given intraperitoneally as a single shot 24 h after the inoculation of sarcoma 180 (1×10^6) into ICR mice. *b*) Tumor cells were collected from mice on day 7.

TABLE III. Antitumor Effect of CO-N on Various Ascitic Tumors

Tumor[a]	Host	Dose[b] (mg × d)	MST[c] (d)	ILS (%)
Ehrlich (1×10^6)	ICR	0.25×10	14.5	16
		1.00×10	32.3	158
		5.00×10	35.8	186
		SN-C (5.00×10)	30.3	142
		Control	12.5	—
		0.50×5	13.8	15
		2.00×5	14.5	20
		10.00×5	28.5	138
		SN-C (10.00×5)	16.5	39
		Control	12.0	—
IMC (1×10^5)	CDF$_1$	10.00×10	59.7	223
		20.00×10	60.0	224
		Control	18.5	—
L1210 (1×10^5)	DBA/2	10.00×10	10.5	0
		20.00×10	11.0	5
		Control	10.5	—
P388 (1×10^5)	DBA/2	10.00×10	18.5	19
		20.00×10	20.0	29
		Control	15.5	—

a) Tumor cells were inoculated intraperitoneally into mice on day 0. *b*) CO-N was given intraperitoneally for 5 or 10 consecutive days after tumor inoculation. *c*) The mortality of mice was recorded for 60 d after tumor inoculation.

Table IV. Antitumor Effect of CO-N on Ehrlich Solid Tumor

Route	Dose[a] (mg/kg × d)	Tumor weight[b] (g) (mean ± S.D.)	Inhibition ratio (%)	Complete cure rate (%)
i.v.	0.12 × 10	1.80 ± 0.55	0	0 (0/9)
	0.50 × 10	2.00 ± 0.67	0	0 (0/7)
	2.0 × 10	1.80 ± 0.48	0	0 (0/9)
	Control	1.76 ± 0.53	—	0 (0/10)
i.p.	5.0 × 10	1.85 ± 0.38	2	0 (0/10)
	10.0 × 10	1.65 ± 0.42	12	0 (0/10)
	100.0 × 10	1.45 ± 0.71	23	10 (1/10)
	Control	1.88 ± 0.63	—	0 (0/10)
i.t.	0.5 × 10	0.79 ± 0.32	11	30 (3/10)
	5.0 × 10	0.44 ± 0.15	51	50 (5/10)
	10.0 × 10	0.15 ± 0.33	83	80 (8/10)
	Control	0.89 ± 0.32	—	0 (0/10)
i.m.	0.5 × 10	1.77 ± 0.53	3	0 (0/10)
	5.0 × 10	1.74 ± 0.71	5	0 (0/10)
	10.0 × 10	1.47 ± 0.62	20	10 (1/10)
	Control	1.83 ± 0.33	—	0 (0/10)

Ehrlich carcinoma cells (1×10^6) were inoculated subcutaneously at the right inguinal region of ICR mice on day 0. *a*) CO-N was given intravenously, intraperitoneally, intramuscularly or intratumorally to mice for 10 consecutive days after tumor inoculation. *b*) Tumors were excised from mice and weighed on day 30.

Table V. Antitumor Effect of CO-N on MM46 Carcinoma Solid Tumor

Route	Dose[a] (μg/kg × d)	Tumor weight[b] (g) (mean ± S.D.)	Inhibition ratio (%)	Complete cure rate (%)
i.v.	1.0 × 5	0.08 ± 0.02	93	70 (7/10)
	10.0 × 5	0.04 ± 0.67	96	80 (8/10)
i.p.	1.0 × 5	0.77 ± 0.35	27	30 (3/10)
	10.0 × 5	0.75 ± 0.22	29	30 (3/10)
	Control	1.06 ± 0.25	—	0 (0/10)

MM46 carcinoma cells (1×10^6) were inoculated subcutaneously at the right groin of C3H/He mice on day 0. *a*) CO-N was given intravenously or intraperitoneally on days 6, 8, 10, 12 and 14 after tumor inoculation. *b*) Tumors were excised from mice and weighed on day 30.

transplantation. As shown in Table III, the life-prolonging effect of CO-N on Ehrlich carcinoma was higher when a given dose was administered every day compared to that observed when a twofold dose was administered every other day. A higher effect was seen with CO-N than the same dose of SN-C. In regard to syngeneic tumors, CO-N showed little life-prolonging effect in mice inoculated with L1210 and P388 whereas the ILS was as high as 224% in the case of IMC tumor.

c) Inhibitory Effect of CO-N against Solid Tumors: When 0.5—100 mg/kg of CO-N was administered to mice, an inhibitory effect was observed against Ehrlich carcinoma by the intratumor administration of only 5 and 10 mg/kg of CO-N, and the inhibition ratios were 50.6, and 83.1%, respectively (Table IV). However, no inhibitory effect was observed by intravenous, intraperitoneal or intramuscular administration. Against MM46 carcinoma, intravenous administration of CO-N at doses of 1 or 10 μg/kg resulted in significant inhibition of tumor growth with an inhibition ratio of over 90% (Table V).

d) Cytocidal Effect of CO-N against Cultured Cells: The direct effect of CO-N on IMC and P388D$_1$ cells *in vitro* are shown in Figs. 2 and 3, respectively. Shortly after the addition of CO-N at doses of 12.5—50 μg/ml, damage to IMC tumor cells was observed. P388D$_1$ cells were cultured

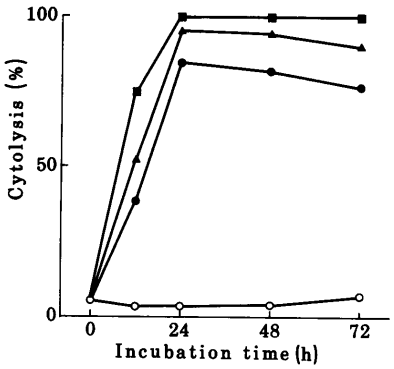

Fig. 2. Cytotoxic Effect of CO-N on IMC Cells *in Vitro*

Tumor cells (2×10^5/ml) were grown in control medium (○), or medium containing 12.5 μg/ml (●), 25 μg/ml (▲) or 50 μg/ml (■) of CO-N.

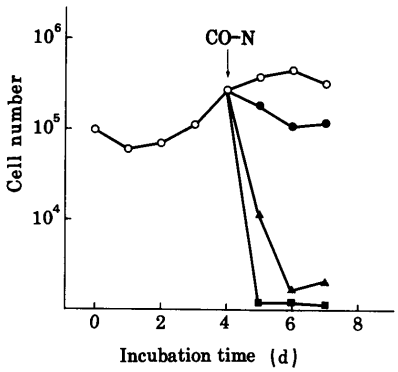

Fig. 3. Cytotoxic Effect of CO-N on P388D$_1$ Cells *in Vitro*

CO-N was added to the culture to give the final concentration of 0 μg/ml (○), 5.0 μg/ml (●), 20.0 μg/ml (▲) or 60.0 μg/ml (■).

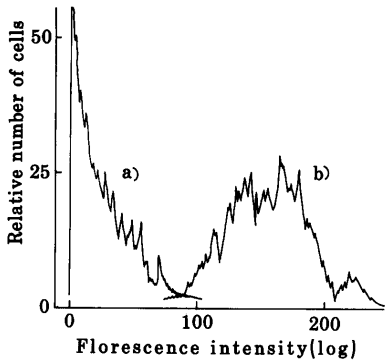

Fig. 4. FACS Analysis of FITC-CO-N Bound to Ehrlich Cells

a) Ehrlich cell-FITC, b) Ehrlich cell-FITC-CO-N.

for 4 d in the absence of CO-N, then various amounts of CO-N were added to the culture. When 5 μg/ml of CO-N was added, a slight growth inhibition was observed. In the culture given 20—60 μg/ml CO-N, a sharp decline in the cell viability was observed and most of the tumor cells has died 48 h after the addition of CO-N.

e) Reactivity of CO-N with Tumor Cell Membrane: The binding of CO-N to tumor cell membrane was determined using a FACS analyzer. It was demonstrated that FITC–CO-N binds to the cell membrane of Ehrlich cells (Fig. 4). Examination under the fluorescence microscope revealed that almost all the cell membranes were stained, and even the nuclei were stained in some of the cells.

Discussion

We have already reported the antitumor activities of a polysaccharide (SN-C) obtained from the culture of *C. ophioglossoides* and a glucan component (CO-1) of SN-C. SN-C possessed both host-mediated and direct effects against various tumors. The host-mediated effect was ascribed to CO-1, consisting of a β(1—3)-glucan main chain with β(1—6) branches.[3,4]

In this study, we investigated the nature of the active moiety of SN-C which exerts the direct effect. A galactosaminoglycan (CO-N) was obtained from the solution after CO-1 had been removed from SN-C. CO-N was a cationic polysaccharide, composed of more than 80% of galactosamine and a small amount of neutral sugars and proteins (Table I). As reported in detail by Yamada *et al.*[10] CO-N is basically α(1—4)-galactosaminoglycans to which neutral sugars and proteins are covalently bound. There have been few reports of galactosamine polymers, and the biological activity of galactosamine polymers is unclear. On the other hand, a glucosamine polymer, chitin, is abundant in nature. Chitosan and chitooligosaccharide are composed of α(1—4)-glucosaminoglycan, and the antitumor activities exerted by these substances are reported to be host-mediated actions such as the activation of macrophages.[2]

CO-N exhibited a potent inhibitory effect on ascitic tumors when given by intraperitoneal administration. Especially against Ehrlich carcinoma, administration of 2 mg/kg of CO-N 10 times resulted in a complete cure. The life-prolonging effect of CO-N against Ehrlich ascitic tumor was greater than that of SN-C. Therefore it can be presumed that the direct effect of SN-C is attributable to the CO-N moiety. In order to elucidate the mechanism of this action, the binding of CO-N to the cell membrane of Ehrlich tumor cells was determined by using FITC–CO-N. The results clearly indicated that CO-N bound to cell membrane. This finding supported the previous assumption that SN-C binds to the tumor cell surface and inhibits the membrane permeability to glucose. It was suggested that the CO-N moiety of SN-C bound to the cell surface and consequently inhibited the proliferation of tumor cells.

CO-N exhibited an inhibitory effect on solid Ehrlich carcinoma only when given intratumorally, and it seemed that the administration of a relatively large dose (1—100 mg/kg) exhibited a direct effect. However, the intravenous administration of CO-N at a low dose (1—10 μg/kg) exhibited a marked inhibitory effect on the tumor growth of MM46 solid tumor, suggesting that the action of CO-N cannot be explained by its direct effect alone.

The addition of SN-C to IMC and P388D$_1$ cultured cells resulted in an inhibition of proliferation without obvious damage to the cells as previously reported.[3] In contrast, CO-N was clearly demonstrated to be cytocidal to these tumor cells. The difference in the direct action on tumor cells between SN-C and CO-N may be explained by their difference in molecular size. The molecular weight of SN-C is very large (700000), while that of CO-N is only 50000. It is plausible that the smaller size of CO-N is advantageous in binding to the surface of tumor cells and penetrating in tumor cells comparing to high molecular weight SN-C. Although the biological activities of CO-N have not yet been fully elucidated, it seems that the administration of a relatively high dose produces a strong direct action through binding to the cell membrane. On the other hand, the administration of a low dose which causes no apparent toxicity to the host is considered to have an indirect antitumor effect. The host responses to a low dose of CO-N should be analyzed.

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