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Dissociation of a Glucan Fraction (CO-1) from Protein-Bound Polysaccharide of *Cordyceps ophioglossoides* and Analysis of Its Antitumor Effect

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A glucan moiety (CO-1) was dissociated from the antitumor protein-bound polysaccharide (SN-C) obtained from the culture of *Cordyceps ophioglossoides* by ultrasonication and heat treatment. CO-1 exhibited antitumor activity against murine sarcoma 180 when given by oral administration. CO-1 was also effective against syngeneic MM46 mammary carcinoma (solid form), but did not show any effect on ascitic tumors such as P388 leukemia. On intraperitoneal administration to normal mice, CO-1 increased the number of peritoneal exudate cells and strongly enhanced the chemiluminescence response of these exudate cells. The activity of CO-1 was higher than that of zymosan-A.

Keywords——*Cordyceps ophioglossoides*; SN-C; ultrasonication; glucan; CO-1; antitumor activity; sarcoma 180; oral administration; chemiluminescence; zymosan-A

Introduction

Many antitumor glucans obtained from microorganisms have been reported.[1] Among them, Lentinan and Schizophyllan, composed mainly of β(1—3) glucans, have already been approved for clinical use in combination with chemotherapeutic agents or radiation.

Previously we have reported that a polysaccharide produced in the culture of *Cordyceps ophioglossoides*, which belongs to *Ascomycetes*, exhibited antitumor activity against sarcoma 180 solid-type tumor and syngeneic ascitic tumors such as MM46 mammary carcinoma. This polysaccharide (SN-C) is homogeneous as judged by gel filtration or ultracentrifugation, consisting mainly of glucan and galactosamine with a small amount of protein. SN-C seems to be a new type of antitumor polysaccharide showing both host-mediated and direct antitumor effects.[2,3] We have now succeeded in the isolation of the glucan moiety (CO-1) by the ultrasonication and heating of SN-C. The present paper describes the isolation of CO-1 and its biological activity.

Materials and Methods

Isolation of Glucan Moiety——At the initial step of isolation of glucans, 500 mg of SN-C, which is water-insoluble, was dissolved in 100 ml of 0.01 N acetic acid (pH 5.8). This solution was exposed to ultrasonic waves generated at 200 W output at 20 °C for 0, 15, 30, 60 and 90 min, using an ultrasonic generator (Kubota Insonator model 200 M, Kubota Ltd., Tokyo, Japan). Then the SN-C solution was heated at 37 °C for 24 h, or at 60 °C for 10,

30 or 60 min, and the resulting precipitates were collected. Each precipitate was analyzed for sugar composition to determine the yield. Hexose content was measured by the phenol–sulfuric acid method,[4] aminosugar content by the indole–hydrochloric acid method,[5] and protein content by the Lowry–Folin method.[6]

Preparation of the Sample——The glucan moiety (CO-1), obtained by ultrasonication (60 min) followed by heating (37 °C, 24 h or 60 °C, 30 min), was dissolved in 0.1 N sodium hydroxide, then neutralized with hydrochloric acid and the osmolarity was adjusted with sodium chloride. The viscosity was decreased by ultrasonication and the solution was sterilized by passage through a 0.45 μm Mirex filter (Millipore Co., Bedford, MA., U.S.A.). The solution was diluted with physiological saline for experimental use in animals and cultured cells. For oral administration, two types of CO-1 preparation were employed. CO-1 powder (100—200 mesh) was suspended in physiological saline and is referred to as CO-1 suspension. Another preparation, CO-1 solution, was prepared by subjecting CO-1 suspension to vigorous ultrasonication (200 W, 120 min).

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, BALB/c, and C57BL/6 mice (7 weeks of age, 20—25 g) were purchased from Charles River Japan, Inc.

Tumors——Sarcoma 180, Ehrlich carcinoma and MM46 mammary carcinoma were kindly provided by the National Cancer Center, and L1210 leukemia, EL4 lymphoma, Meth-A fibrosarcoma and X5563 plasmacytoma by the National Defense Medical College. Each tumor was maintained in the peritoneal cavity of inbred mice by serial passages.

Determination of Antitumor Effects——Effect against Solid Tumors: Animals, tumors and the number of transplanted cells used in the study were as follows: ICR (sarcoma 180, 1×10^6), BALB/c (Meth-A, 2×10^5), and C3H/He (X5563, 2×10^6). Tumor cells were inoculated subcutaneously into the right inguinal region of mice. CO-1 was administered intraperitoneally or orally for 16 consecutive days from 5 d prior to tumor transplantation or for 10 consecutive days from 1 d after the transplantation. The tumor was excised 30 d after the transplantation and the weight was measured and compared with that of the saline-treated 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-1-treated group and C is that of the saline-treated group.

Effect on Ascitic Tumors: Animals, tumors and the number of transplanted cells used in the study were as follows: ICR (Ehrlich, 1×10^5), DBA/2 (P388, 1×10^5), DBA/2 (L1210, 1×10^5) and C57BL/6 (EL4, 2×10^5). The tumor was implanted into the peritoneal cavity of mice, and CO-1 was administered intraperitoneally for 10 consecutive days from 24 h after tumor transplantation. The mortality of mice was observed for 60 d after the transplantation. The increase in life span (ILS) was calculated by comparing the median survival time (MST) of the CO-1-administered group with that of the saline-treated group according to the following formula; ILS (%) $= (T/C - 1) \times 100$, where T is the MST of the CO-1-treated group and C is that of the saline-treated group.

Direct Cytotoxic Effect——Ehrlich carcinoma cells were prepared to a concentration of 1×10^4/ml with RPMI-1640 (10% fetal calf serum (FCS)), and CO-1 was added to give final concentrations of 50, 200 or 500 μg/ml. Then the cells were cultured in a CO_2 incubator at 37 °C for 24, 48 and 72 h. After the incubation, viable cells were counted by the trypan blue dye exclusion method.

Effect on Peritoneal Cells——CO-1 was administered at a dose of 5 mg/kg into the peritoneal cavity of normal female ICR or BALB/c mice. The cells in the peritoneal cavity of mice were collected with Hanks' balanced salt solution 1—7 d after the administration, and the number of peritoneal exudate cells (PECs) in the cavity was counted by the trypan blue dye exclusion method. Differential white cell counts were determined by microscopic examination of the smear of PECs after fixation in methanol and staining with Giemsa stain.

Luminol-Dependent Chemiluminescence——PECs obtained from mice administered with CO-1 were suspended to give 3×10^6/ml in buffer 1 (KCl, 5 mM; NaCl, 145 mM; glucose, 5.5 mM; 10 mM *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES), pH 7.4). The solution was incubated at 37 °C for 5 min, mixed with an equal volume of Chemilight (*N*-formyl-L-methionyl-L-leucyl-L-phenylalanine), and re-incubated for another 5 min. Immediately after the addition of buffer 2 (1 mM $CaCl_2$ in buffer 1) and the luminol solution (Lumilight), the chemiluminescence was measured with an ATP-Photometer (Monolight 401; Analytical Luminescence Laboratory Inc., U.S.A.).

Results

Isolation of Glucan by Physicochemical Treatment of SN-C

SN-C was not soluble in dilute hydrochloric acid or sulfuric acid, but was soluble in organic acids such as acetic acid, citric acid and lactic acid. As shown in Table I, when SN-C solution in dilute acetic acid was ultrasonicated for over 60 min, the amount of precipitates remained constant and the yield was as high as 300 mg. This yield represented 80% recovery of glucose contained in the starting amount of SN-C. Each precipitate obtained under various

TABLE I. Properties of Precipitates Obtained from Ultrasonicated SN-C

Sonication time (min)	Yield[a] (mg)	Component (%)		
		Hexose[b]	Hexosamine[c]	Protein[d]
0	0	—	—	—
15	124.1	93.0	1.3	1.5
30	195.5	92.1	1.5	2.0
60	304.6	95.5	nd	nd
90	308.3	95.3	nd	nd
SN-C	—	75.2	17.3	5.5

a) SN-C (500 mg) was dissolved in 100 ml of 0.01 N acetic acid and treated by ultrasonication for various times. After heating at 60 °C for 30 min, the resulting precipitates were measured to determine the yields. *b*) Hexose content was measured by the phenol–sulfuric acid method. *c*) Hexosamine content was measured by the indole–hydrochloric acid method. *d*) Protein was measured by the Lowry–Folin method. nd, not detectable.

TABLE II. Effect of Heating Conditions on Separation of Glucan

Heating temp.–time	Yield[a] (mg)	Component (%)		
		Hexose[b]	Hexosamine[c]	Protein[d]
37 °C–24 h	295.4	95.3	nd	nd
60 °C–10 min	270.3	95.0	nd	nd
60 °C–30 min	304.6	95.5	nd	nd
60 °C–60 min	312.8	92.3	2.5	1.5
SN-C	—	75.2	17.3	5.5

a) SN-C (500 mg) was dissolved in 100 ml of 0.01 N acetic acid. *b*) Hexose content was measured by the phenol–sulfuric acid method. *c*) Hexosamine content was measured by the indole–hydrochloric acid method. *d*) Protein was measured by the Lowry–Folin method. nd, not detactable.

conditions contained more than 90% glucose and it was glucan with a high purity. The protein content was extremely low in every case. Glucans were precipitated by heating at 37 °C, but a longer time was required for complete precipitation (Table II). However, when the heating was carried out at 60 °C for 60 min after ultrasonication, the glucose content of the precipitate was relatively decreased and a small amount of galactosamine and protein newly appeared (Table II). On the basis of these results, the subsequent isolation of CO-1 was performed by the ultrasonication of SN-C for 60 min followed by heating at 37 °C for 24 h or at 60 °C for 30 min.

Inhibitory Effect of CO-1 on Solid Tumor Growth

In the previous report, it was shown that administration of 5 mg/kg of CO-1 ten times to mice *via* the intraperitoneal or intravenous route produced almost 100% growth inhibition of a solid-type tumor, sarcoma 180.[7] The effect of oral administration of CO-1 against sarcoma 180 is shown in Table III. The oral administration of CO-1 solution which was prepared by ultrasonication at doses of 5—100 mg/kg for 10 d exhibited an inhibition, although weak, of the tumor growth. When CO-1-solution was administered before the tumor transplantation, a similar antitumor effect was observed. However, the suspension of CO-1 showed no inhibitory effect on tumor growth even at doses of 10—250 mg/kg. The effects against syngeneic tumors are summarized in Table IV. The intraperitoneal administration of 10 mg/kg of CO-1 suppressed the growth of MM46 carcinoma with an inhibition ratio of 62.2%. CO-1 showed no effect on the growth of Meth-A or X5563 tumors.

TABLE III. Antitumor Effect of CO-1 with Various Treatment Schedules on Sarcoma 180 Solid Tumor

Sample	Dose[a] (mg/kg)	Treat. schedule (d × time)	Tumor weight[b] (g) (mean ± S.D.)	Inhibition ratio (%)
CO-1 suspension[c]	10	+1—+10×10	1.25±0.73	0
	100	+1—+10×10	1.30±0.52	0
	250	+1—+10×10	1.55±0.43	0
	10	−5—+10×16	1.28±0.28	0
	100	−5—+10×16	1.35±0.55	0
	250	−5—+10×16	1.20±0.63	2
CO-1 solution[d]	5	+1—+10×10	0.92±0.25	25
	50	+1—+10×10	0.98±0.32	20
	100	+1—+10×10	0.97±0.43	21
	5	−5—+10×16	0.90±0.25	27
	50	−5—+10×16	0.67±0.15	46
	100	−5—+10×16	0.95±0.50	23
Control			1.23±0.33	

Sarcoma 180 cells (5×10^5) were inoculated subcutaneously at the right inguinal region of ICR mice on day 0. *a*) CO-1 was administered orally on the indicated days after tumor inoculation. *b*) Tumors were excised from mice and weighed on day 30. *c*) CO-1 suspension was prepared by suspending CO-1 powder (100—200 mesh) in physiological saline. *d*) CO-1 solution was prepared by ultrasonication (200 W, 120 min) of CO-1 suspension.

TABLE IV. Antitumor Effect of CO-1 on Syngeneic Solid Tumors

Tumor[a]	Host	Dose[b] (mg/kg × d)	Tumor weight[c] (g) (mean ± S.D.)	Inhibition ratio (%)
Meth-A	BALB/c	2.5×10	3.30±0.78	0
		5.0×10	3.25±0.56	0
		10.0×10	3.18±0.33	0
		25.0×10	3.18±0.48	0
		Control	3.19±0.55	
X5563	C3H/He	2.5×10	3.21±0.63	13
		5.0×10	3.69±0.72	0
		10.0×10	3.77±0.83	0
		Control	3.67±0.48	—
MM46	C3H/He	2.5×10	2.31±0.73	18
		5.0×10	1.88±0.62	34
		10.0×10	1.07±0.44	62
		25.0×10	1.55±0.53	45
		Control	2.83±0.32	—

a) Tumor cells (2×10^5) were inoculated subcutaneously at the right inguinal region of each mouse on day 0. *b*) CO-1 was given intraperitoneally for 10 consecutive days after tumor inoculation. *c*) Tumors were excised from mice and weighed on day 30.

Effect of CO-1 on the Life Span (Survival Time) of Mice Bearing Ascitic Tumors

The life-prolonging effect in mice with each tumor is presented in Table V. The intraperitoneal administration of CO-1 at doses of 2.5—10.0 mg/kg exhibited little life-prolonging effect in mice with Ehrlich carcinoma, L1210, P388 and EL4.

Inhibitory Effect on the Proliferation of Cultured Cells

The proliferation of cultured Ehrlich cells was not inhibited by the addition of CO-1 even at doses of 50—500 μg/ml (data not shown).

TABLE V. Antitumor Effect of CO-1 on Various Ascitic Tumors

Tumor[a] (Cell number)	Host	Dose[b] (mg × d)	MST[c] (d)	ILS (%)
Ehrlich (1×10^6)	ICR	2.5×10	17.0	6
		5.0×10	19.0	19
		10.0×10	18.0	13
		Control	16.0	—
L1210 (1×10^4)	DBA/2	2.5×10	11.5	10
		5.0×10	11.0	5
		10.0×10	12.0	14
		Control	10.5	—
P388 (1×10^4)	DBA/2	2.5×10	16.0	3
		5.0×10	16.0	3
		10.0×10	16.5	7
		Control	15.5	—
EL4 (1×10^4)	C57BL/6	2.5×10	15.0	0
		5.0×10	17.0	13
		10.0×10	15.5	3
		Control	15.0	—

a) All tumors cells were inoculated intraperitoneally into mice on day 0. *b*) CO-1 was given intraperitoneally for 10 consecutive days after tumor inoculation. *c*) The mortality of mice was recorded for 60 d after tumor inoculation.

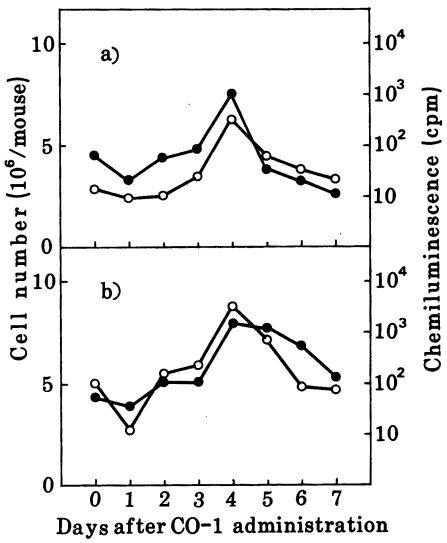

Fig. 1. Kinetics of Peritoneal Exudate Cell Number and Activity to Emit Chemiluminescence in Mice Treated with CO-1

Mice were intraperitoneally injected with 5 mg/kg. of CO-1. Peritoneal exudate cells were collected from each mouse on days 1, 2, 3, 4, 5, 6 and 7 after administration of CO-1. The cell numbers were counted using a hemocytometer. (○), cell numbers; (●), fluorescence intensity. a), BALB/c mice; b), ICR mice.

Changes in the Number of Peritoneal Exudate Cells (PECs) and the Activity to Emit Chemiluminescence

The number of PECs before CO-1 administration was 2.6×10^6 in BALB/c mice and 5×10^6 in ICR mice. Four days after the intraperitoneal administration of CO-1, the number was increased in both strains of mice to 5.8×10^6 and 7.5×10^6, respectively. Chemiluminescence by PECs was also significantly augmented in both strains on day 4 of CO-1 administration (Fig. 1). The kinetic change of chemiluminescence was similar to that of PEC number. A more than 10-fold increase was observed in the chemiluminescence by PECs 4 d after CO-1 administration compared with non-stimulated PECs, and it was suggested that these macrophages are in an activated state. Differential white cell counts of PECs revealed that lymphocytes predominated before CO-1 administration and that the number of

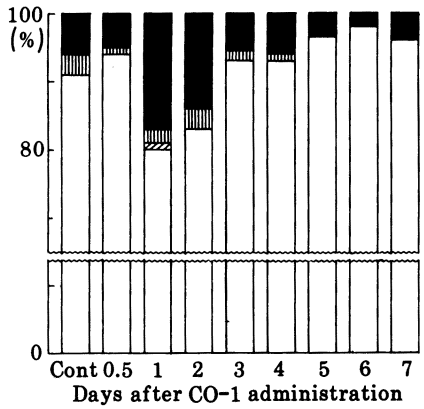

Fig. 2. Kinetics of Peritoneal Exudate Cells in Mice Treated with CO-1

CO-1 was given intraperitoneally to ICR mice. Peritoneal exudate cells were collected from each mouse on days 0.5, 1, 2, 3, 4, 5, 6 and 7 after administration of CO-1.

Cont, control mice; (■), macrophages; (▥), neutrophils; (▨), eosinophils; (□), lymphocytes.

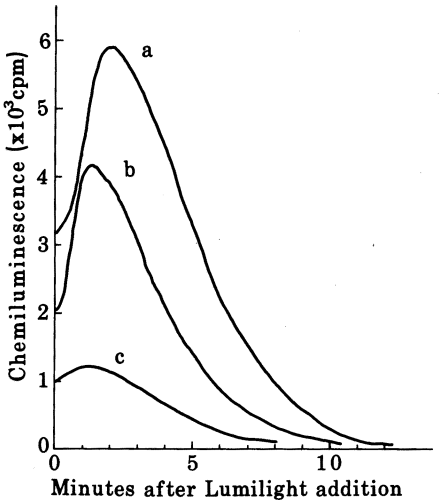

Fig. 3. The Activity to Emit Chemiluminescence of PECs from Mice Given CO-1 or Zymosan-A

CO-1 was given intraperitoneally to C57BL/6 mice on day 0. Peritoneal exudate cells were collected from each group of mice on day 4.

a), CO-1 (5 mg/kg); b), zymosan-A (50 mg/kg); c), saline.

macrophages was increased 1 to 2 d after the administration (Fig. 2). The enhancement of chemiluminescence by CO-1 was compared with that by zymosan-A (Sigma Chemical Co., St. Louis, MO., U.S.A.). The chemiluminescence emitted by PECs 4 d after the intraperitoneal administration of CO-1 at a dose of 5 mg/kg to mice was much higher than that by PECs from mice obtained 4 d after administration of 50 mg/kg of zymosan-A (Fig. 3).

Discussion

We have already reported the antitumor effect of a polysaccharide (SN-C) obtained from the culture of *C. ophioglossoides*. SN-C is a protein-bound polysaccharide composed of glucose and galactosamine as main sugar components and considered to be homogenous in terms of gel filtration and ultracentrifugation.[3] For the purpose of further fractionation of SN-C, we examined various methods such as ion exchange chromatography, alcohol precipitation, electophoresis or affinity chromatography without satisfactory results. SN-C is insoluble in water, but soluble in dilute organic acids such as acetic acid, citric acid and lactic acid. Therefore, we attempted the ultrasonic treatment of a dilute acetic acid solution to dissociate the glucan moiety. We found that highly purified glucans could be precipitated by heating the sonicated sample of SN-C solution. The molecular weight of SN-C is 700000, whereas that of CO-1 was 632000 as estimated by gel filtration.[2,7]

Although the mechanism of the precipitate formation by the present treatments is not known, it is assumed that glucan precipitates after cleavage of the linkage region between the glucan and the protein moiety or between the glucan and the galactosaminoglycan moiety as a result of the sonication and the subsequent heating in dilute acetic acid. Alternatively, the precipitate formation may be a result of a change of the tertiary structure of the glucan when it is free of protein and galactosaminoglycan.

Previously, we reported the detailed structure of CO-1. CO-1 is a glucan consisting of a

backbone of β(1—3)-D-glucans having β(1—6)-linked branches, distributed mainly in the non-reducing and reducing terminals.[8] Many antitumor glucans so far isolated from various living organisms, including Lentinan and Schizophyllan, are 6-branched β(1—3)-glucans.[1] While most of the glucans do not exhibit antitumor activity on oral administration, there is a glucan which is reported to be effective against solid tumors such as sarcoma 180. This glucan, effective by oral administration, has an α-linkage.[9] The difference in the antitumor activity of various glucans may be attributable to differences in the primary structures of glucans. In this study, the inhibition of tumor growth was observed in the group to which CO-1 solution was given orally, whereas no effect was seen in the group given the suspension form. This suggests that a possible difference in intestinal absorption between solution and suspension may result in the different expressions of antitumor effect. There is a report that water-insoluble β(1—3)-glucans with (1—6)-linked side chains have no antitumor activity.[10] Although the glucan we obtained in the present study was soluble in alkaline solution and insoluble in water, its antitumor activity was similar to those of the other antitumor glucans reported previously.[1]

The number of peritoneal exudate cells in mice was increased by the intraperitoneal administration of CO-1. The increase in number reached its peak in 4 d after administration, and chemiluminescence was markedly elevated. The inhibitory effect of CO-1 on the *in vivo* growth of some syngeneic tumors may be ascribed to such a functional activation of macrophages, since it is reported that the level of chemiluminescence emitted by macrophages is closely related to the antitumor activity.[11] The reason for the ineffectiveness of CO-1 against ascitic tumors is not clear. Administration of CO-1 before the inoculation of ascitic tumor cells might result in a protective effect.

In a separate paper, we have shown that SN-C has both host-mediated and direct antitumor effects. CO-1, the glucan moiety of SN-C, showed antitumor activity *in vivo* without showing any inhibition of the tumor cell proliferation *in vitro*. The results of this study clearly demonstrated that one of the two effects, the host-mediated action, is attributable to CO-1.

In the future, we are planning to evaluate the immunological activities of CO-1 and to investigate the active site of SN-C which is responsible for the direct effect.

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