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Antimetastatic and Growth-inhibitory Effects of *N*-Acetylchitohexaose in Mice Bearing Lewis Lung Carcinoma

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N-Acetylchitohexaose, a water-soluble oligosaccharide was found to display a significant antimetastatic effect against Lewis lung carcinoma (LLC) transplanted into C57BL/6 mice, giving rise to a 40–50% inhibition ratio of pulmonary metastasis when administered intravenously (1 mg/kg) on day 6 after the tumor implantation (5×10^5 cells/mouse). It was also revealed that this hexaose had a significant growth-inhibitory effect against the local tumor of the same carcinoma (a 20–30% inhibition ratio), showing an enhancing effect on concomitant immunity in local tumor-resected mice. This oligosaccharide was also shown to enhance the tumoricidal effect of splenic T lymphocytes against LLC and P-815 mastocytoma cells and to increase the natural killer activity of splenic T lymphocytes, assayed with YAC-1 cells as the target.

Key words: Lung metastasis — *N*-Acetylchitohexaose — Lewis lung carcinoma

In the preceding papers of this series, we reported that, *N*-acetylchitohexaose (NACOS-6) and chitohexaose (COS-6), water-soluble lower homologs of chitin and chitosan,[1] respectively, were able to enhance the immunological defense mechanism of mice in terms of antitumor and antimicrobial effects, when these oligosaccharides were administered intravenously.[2–6] With regard to the mechanisms of action of NACOS-6 and particularly COS-6, we have provided evidence of the facilitation of the defense functions of macrophages (Mϕ), polymorphonuclear leukocytes (PMN), cytotoxic T (CTL), and natural killer (NK) cell activities due to the induction of interleukins 1 and 2 (IL-1 and -2).[7,8] As a next step, we have conducted an antimetastasis assay of NACOS-6 on Lewis lung carcinoma (LLC) transplanted into C57BL/6 mice.

MATERIALS AND METHODS

Animals C57BL/6 male mice, 5 to 6 weeks of age were obtained from the Shizuoka Agricultural Cooperation for Experimental Animals, Hamamatsu. These mice were housed six/cage in air-conditioned quarters, and were provided food and water *ad libitum*.

Tumor cells The Lewis lung carcinoma (LLC) was obtained from the Research Institute for Tuberculosis and Cancer, Tohoku University Sendai. This tumor was maintained continuously in our laboratory in solid form in C57BL/6 mice. YAC-1, LLC and P-815 cells were used as targets for measuring cell cytotoxic activity. Each tumor line was maintained by *in vitro* culture in RPMI-1640 medium containing 10% NBS.

N-Acetylchitohexaose (NACOS-6) This oligosaccharide was supplied by Ihara Chemical Industries, Tokyo. The lipopolysaccharide content of NACOS-6 was below 10 pg/mg as assayed by the method of Morita *et al.*[9]

Lentinan Lentinan was kindly provided by Ajinomoto Co., Ltd., Tokyo.

Recombinant murine interferon-γ This was kindly provided by Dr. S. Kobayashi, Toray Industries, Inc., Kanagawa.

Antitumor and antimetastatic assay For the test of antitumor assay, 7-day-old LLC tumor cells (5×10^5) were inoculated into mice by subcutaneous implantation in the right groin and the animals were randomly divided into experimental groups of 10 to 12 mice each. The sample was given intravenously as a single dose of 0.1, 1, 10, or 100 mg/kg on day 6. The effect on the local tumor and its metastatic spread in the lungs was evaluated using the method of Wexler[10] and the assays were repeated five times.

Concomitant immunity assay In order to analyze the effect of NACOS-6 on concomitant immunity for post-surgical metastasis, C57BL/6 mice were implanted with 5×10^5 cells of LLC into the footpad and randomly divided into experimental groups of 12 mice each. The primary tumor was resected on day 12. Mice were treated with NACOS-6 intravenously, 7, 12, and 17 days after the tumor inoculation. The lungs of the mice were weighed on day 24. This assay was conducted with 12 mice/group and repeated four times.

Preparation of effector cells Effector cells consisting of whole spleen cells or spleen cells depleted of macrophages

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and B lymphocytes were used. In the latter case, macrophages and B lymphocytes were removed from effector spleen cells by passage through a nylon wool column.[11] After the passage, complete or nonadherent T lymphocytes (2×10^5 to 2×10^6) were assayed for cell-mediated cytotoxicity.

Cell-mediated cytotoxicity assay The LLC, P-815 and YAC-1 cells were used as target cells. In round-bottomed wells of 96-well plates (Corning Glass Works, N.Y.), 2×10^5 to 2×10^6 effector spleen cells were mixed with 2×10^4 target cells. The cells were incubated for 4 or 18 hrs at 37°C in a humidified atmosphere of 5% CO_2 in air, then viable and dead target cells were counted by the trypan blue dye exclusion method,[12] and the assay was repeated five times.

RESULTS

Antimetastatic effect of NACOS-6 on pulmonary metastasis of LLC in mice As summarized in Table I, treatment of C57BL/6 mice with a single administration of NACOS-6 (1 mg/kg intravenously) on day 6 after tumor implantation resulted in a significant decrease in the number of pulmonary nodules formed by metastasis of this carcinoma (a 40 to 50% inhibition ratio). Other doses, higher or lower than 1 mg/kg, gave lower inhibition ratios, so it is possible to state that the optimal dose is approximately 1.0 mg/kg. Interferon γ (IFN-γ), used as the positive control in this assay, showed a 40% antimetastatic effect at an intramuscular dose of 1,000 U/mouse/day for 14 successive days. These results show that the antimetastatic effect of a single administration of 1 mg/kg NACOS-6 is comparable with that of 1,000 U of IFN-γ/day for 14 days.

Growth-inhibitory activity of NACOS-6 on LLC local tumor growth in mice As shown in Table II, NACOS-6 exhibited low inhibition ratios, less than 30%, against this solid tumor in the dose range from 0.1 to 10 mg/kg. The effect of NACOS-6 is comparable with that of 100 to 1,000 U of IFN-γ.

Enhancing effect of NACOS-6 on concomitant immunity for postsurgical metastases in LLC-bearing mice Table III shows that NACOS-6 exhibited a significant antimetastatic effect. These results indicate that NACOS-6 possesses an enhancing effect on concomitant immunity in local tumor-resected mice.

Table I. Effect of NACOS-6 on the Pulmonary Metastasis of Lewis Lung Carcinoma (LLC) in C57BL/6 Mice[a]

Sample[b]	Dose (/kg)	Number of lung nodules[c] (mean±SE)	Inhibition ratio (%)	P[d] vs. Control	P[d] vs. Lentinan
Experiment 1					
Control		131.8±20.9			
NACOS-6	100 mg×1	94.1±5.1	28.6	ns[e]	ns
	10 mg×1	92.0±9.3	30.2	ns	ns
	1 mg×1	61.3±5.1	53.5	<0.01 (≦0.01)	<0.001 (≦0.01)
Lentinan	2.5 mg×1	97.9±7.6	25.7	ns	
Experiment 2					
Control		54.6±4.4			
NACOS-6	10 mg×1	43.5±6.1	20.3	ns	
	1 mg×1	34.9±4.3	36.1	<0.01 (≦0.01)	
	0.1 mg×1	52.0±6.3	4.8	ns	
Experiment 3					
Control		66.9±14.8			
IFN-γ	10,000 U×14	48.6±20.2	27.4	<0.05 (0.01<P≦0.05)	
	1,000 U×14	38.6±26.5	42.3	<0.05 (0.01<P≦0.05)	
	100 U×14	53.8±12.7	19.6	ns	
NACOS-6	1 mg×1	37.2±14.2	44.4	<0.01 (≦0.01)	
	0.1 mg×1	41.4±14.7	38.1	<0.05 (0.01<P≦0.05)	

a) Mice were implanted with 5×10^5 cells of LLC.
b) Mice were treated intravenously with NACOS-6 or lentinan 6 days after the tumor inoculation. IFN-γ was administered intramuscularly at a dose of 100 to 10,000 U/day for 14 successive days.
c) The number of lung nodules was counted on day 21.
d) Student's t test (Mann Whitney U test).
e) ns: not significant.

Table II. Effect of NACOS-6 on LLC Local Tumor Growth in C57BL/6 Mice[a]

Sample[b]	Dose (/kg)	Tumor weight[c] (g) (mean±SE)	Inhibition ratio (%)	P[d] vs. Control
Experiment 1				
Control		8.22±0.39		
NACOS-6	100 mg×1	7.05±0.62	14.3	ns[e]
	10 mg×1	6.80±0.34	17.3	<0.05
	1 mg×1	6.42±0.51	22.0	<0.05
Lentinan	2.5 mg×1	6.18±0.34	24.8	<0.001
Experiment 2				
Control		6.16±1.25		
IFN-γ	10,000 U×14	4.92±1.88	20.3	ns
	1,000 U×14	3.74±2.52	39.3	<0.05
	100 U×14	3.68±2.04	40.3	<0.05
NACOS-6	1 mg×1	4.27±2.25	30.7	<0.05
	0.1 mg×1	3.71±2.04	40.0	<0.05

a) Mice were implanted with 5×10^5 cells of LLC.
b) Mice were treated intravenously with NACOS-6 or lentinan 6 days after the tumor inoculation. IFN-γ was administered intramuscularly at a dose of 100 to 10,000 U/day for 14 successive days.
c) The tumor weight was measured on day 21.
d) Student's t test.
e) ns: not significant.

Table III. Effect of NACOS-6 on Concomitant Immunity for Postsurgical Metastases in LLC-bearing C57BL/6 Mice[a]

Sample[b]	Dose (mg/kg)	Lung weight[c] (mg) (mean±SE)	Inhibition ratio (%)	P[d] vs. Control
Control		487.6±80.3		
NACOS-6	10×3	565.3±144.6	−15.9	ns[e]
	1×3	528.3±109.3	−8.3	ns
	0.1×3	285.2±36.2	41.5	<0.05
Lentinan	10×1	588.0±72.0	−20.5	ns

a) Mice were implanted with 5×10^5 cells of LLC into the footpad and the primary tumor was resected on day 12.
b) Mice were treated intravenously with NACOS-6 7, 12 and 17 days after the tumor inoculation.
c) The lungs of mice were weighed on day 24.
d) Student's t test.
e) ns: not significant.

Enhancing effect of NACOS-6 on tumoricidal activity of spleen lymphocytes against tumor target cells In order to analyze the action mechanism of NACOS-6, cytotoxic T cell activity was estimated with LLC and P-815 mastocytoma as the target cells. As summarized in Table IV, nylon wool column-passed spleen cells from tumor-bearing mice pretreated with NACOS-6, 1 to 100 mg/kg, exhibited an increased cytolytic effect against LLC, i.e., a 100:1 mixture of the splenic T cells pretreated at 1 mg/kg and LLC showed a cytolytic ratio approximately 2.6 times higher than that of the untreated control group. Because a good proportional relationship exists between the three ranges of E:T ratios and the percent of dead target cells, it is likely that a large number of T cells among the whole spleen cells participate in this effect, and that activation of a fairly large quantity of T cells in mice has been achieved by treatment with NACOS-6. Another cytolysis assay using P-815 mastocytoma cells gave more distinct results concerning the relationship between E:T and cytolysis ratios (Table V). Namely,

Table IV. Effect of NACOS-6 Treatment on Tumoricidal Activity of Splenic T Lymphocytes from LLC-bearing C57BL/6 Mice[a] against LLC Cells

Mice injected with[b]	Dose (mg/kg×1)	E:T[c]	Percent of dead target cells (mean±SE)	P[d] vs. Control
Control		100:1	10.3±2.2	
NACOS-6	1		26.2±2.8	<0.01
Lentinan	2.5		22.1±0.6	<0.01
Control		50:1	8.5±1.3	
NACOS-6	1		18.5±1.8	<0.01
Lentinan	2.5		14.8±1.6	<0.05
Control		10:1	6.7±1.7	
NACOS-6	1		6.2±2.3	ns[e]
Lentinan	2.5		5.8±1.0	ns
Control		100:1	9.0±1.4	
NACOS-6	100		43.4±2.3	<0.001
	10		nd[f]	
Lentinan	2.5		51.9±2.4	<0.001
Control		50:1	6.8±0.8	
NACOS-6	100		18.4±0.6	<0.001
	10		nd	
Lentinan	2.5		18.9±0.5	<0.001
Control		10:1	6.8±1.2	
NACOS-6	100		6.6±1.8	ns
	10		nd	
Lentinan	2.5		8.5±0.6	ns

a) Mice were implanted with 5×10^5 cells of LLC.
b) Mice were treated intravenously with NACOS-6 on day 6 after the tumor implantation. Ten days after the tumor inoculation, the tumoricidal activity of splenic T lymphocytes was assayed. The number of dead target cells was counted by the trypan blue dye exclusion method.
c) E: Effector cells (nylon wool column-passed spleen cells). T: Target cells (LLC cells).
d) Student's t test.
e) ns: not significant.
f) nd: not done.

higher E:T ratios (100:1 and 50:1) resulted in larger percentages of dead target cells as compared with those in the cytolysis assay using LLC as the target cells.

Enhancing effect of NACOS-6 on natural killer activity

NACOS-6 was also found to enhance NK activity as determined with YAC-1 lymphoma cells as the target, although the elevation of this activity was not as great as that observed on the tumoricidal effect of cytolytic T cells (Table VI).

DISCUSSION

One of the most serious problems of cancer treatment is the metastasis of local tumors to other organs and tissues. The manifestation of antimetastatic properties has therefore been regarded as an essential feature of cancer therapeutic agents.[13-15]

In the previous paper, we revealed that NACOS-6, one of the water-soluble and low-molecular homologs of *N*-acetylchitooligosaccharides consisting solely of β-1,4-linked *N*-acetyl-D-glucosamine, exhibited antitumor and antimicrobial activities.[2-8]

In the present study, we revealed that NACOS-6 was able to manifest antimetastatic and growth-inhibitory effects on Lewis lung carcinoma in mice, and this effect is comparable with that of intramuscular administration of 1,000 U of IFN-γ/day for 14 days. It should be noted that, even at the highest dose, 100 mg/kg, none of the mice showed any tendency for rapid tumor growth, which is not the case using other cancer immunotherapeutic agents, including those of polysaccharide nature. Because the rapid enhancement of tumor growth suggests the elevation of suppressor T cell activity, the lack of this property seems quite favorable for cancer

Table V. Effect of NACOS-6 Treatment on Tumoricidal Activity of Splenic T Lymphocytes from LLC-Bearing C57BL/6 Mice[a] against P-815 Mastocytoma Cells

Mice injected with[b]	Dose (mg/kg×1)	E:T[c]	Percent of dead target cells (mean±SE)	P[d] vs. Control
Control		100:1	12.8±1.8	
NACOS-6	1		41.4±2.0	<0.001
Lentinan	2.5		39.9±3.7	<0.001
Control		50:1	7.3±0.6	
NACOS-6	1		16.9±1.0	<0.001
Lentinan	2.5		19.4±0.7	<0.001
Control		10:1	5.8±1.0	
NACOS-6	1		7.0±1.2	ns[e]
Lentinan	2.5		8.4±0.4	<0.05
Control		100:1	3.3±1.0	
NACOS-6	100		22.5±0.8	<0.001
	10		nd[f]	
Lentinan	2.5		27.8±0.5	<0.001
Control		50:1	1.7±0.4	
NACOS-6	100		11.7±0.7	<0.001
	10		nd	
Lentinan	2.5		11.8±3.4	ns
Control		10:1	3.2±1.1	
NACOS-6	100		2.7±0.4	ns
	10		nd	
Lentinan	2.5		1.6±0.4	ns

a) Mice were implanted with 5×10^5 cells of LLC.
b) Mice were treated intravenously with NACOS-6 on day 6. Ten days after the tumor inoculation, the tumoricidal activity of splenic T lymphocytes was assayed. The number of dead target cells was counted by the trypan blue dye exclusion method.
c) E: Effector cells. T: Target cells (P 815 mastocytoma cells).
d) Student's t test.
e) ns: not significant.
f) nd: not done.

immunotherapeutic agents. NACOS-6 was then investigated for its enhancing effect on concomitant immunity for postsurgical metastases of LLC. NACOS-6 exhibited a significant antitumor effect. In order to analyze the action mechanism of NACOS-6, cytotoxic T cell activity and natural killer activity were estimated. NACOS-6 was found to enhance both activities.

In conclusion, NACOS-6, a simple oligosaccharide consisting solely of *N*-acetyl-D-glucosamine residues, was shown to manifest a significant antimetastatic effect toward LLC implanted in C57BL/6 mice. It is therefore likely that activation of the tumoricidal functions of splenic T cells and NK cells in the spleen of mice is involved in the antimetastatic and antitumor effects.

We have conducted a series of immunological assays on chitin, chitosan, mannan and glucan from the cell wall of bakers' yeast (*Saccharomyces cerevisiae*), including assays of their antitumor and antimicrobial effects,[16-19] and have found that these polysaccharides, as well as other immunopotentiating polysaccharides such as lentinan, schizophyllan and so on, were able to manifest antitumor effects in mice bearing solid tumors. The fact that NACOS-6 and COS-6 were able to display antitumor effects indicates that polymeric structure is not essential for developing this effect. Further studies on the effectiveness of NACOS-6 as an antitumor agent are in progress.

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Table VI. Effect of NACOS-6 Treatment on Natural Killer (NK) Activity of Spleen Cells from C57BL/6 Mice

Mice injected with[a]	Dose (mg/kg×1)	E:T[b]	Percent of dead target cells (mean±SE)	P[c] vs. Control	P[c] vs. Lentinan
Control		100:1	63.2±3.1		
NACOS-6	1		76.9±4.9	<0.05	ns[d]
Lentinan	2.5		75.8±1.1	<0.01	
Control		50:1	39.0±2.0		
NACOS-6	1		49.1±0.8	<0.01	<0.001
Lentinan	2.5		36.2±1.9	ns	
Control		10:1	21.7±1.1		
NACOS-6	1		27.9±0.8	<0.01	<0.001
Lentinan	2.5		20.3±0.9	ns	
Control		100:1	60.3±4.2	<0.05	ns
NACOS-6	100		65.2±5.6	ns	ns
	10		nd[e]		
Lentinan	2.5		73.0±6.2	<0.05	
Control		50:1	38.1±3.9		
NACOS-6	100		47.3±2.6	<0.05	ns
	10		nd		
Lentinan	2.5		43.8±4.8	<0.05	
Control		10:1	20.3±1.8		
NACOS-6	100		26.5±5.2	ns	ns
	10		nd		
Lentinan	2.5		23.4±2.5	ns	

a) Mice were treated intravenously with NACOS-6 on day 0, and NK activity was assayed on day 4. The number of dead target cells was counted by the trypan blue dye exclusion method.
b) E: Effector cells (whole spleen cells). T: Target cells (YAC-1 lymphoma cells).
c) Student's *t* test.
d) ns: not significant.
e) nd: not done.

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