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Growth-Inhibitory Effect of Hexa-*N*-acetylchitohexaose and Chitohexaose against Meth-A Solid Tumor

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Two oligosaccharides, hexa-*N*-acetylchitohexaose (NACOS-6) and chitohexaose (COS-6), consisting solely of *N*-acetyl-D-glucosamine and D-glucosamine, respectively, were found to be growth-inhibitory against Meth-A solid tumor transplanted into BALB/c mice, when each oligosaccharide was administered i.v. at a dose of more than 1 mg/kg. Mouse peritoneal macrophages elicited with casein underwent activation with these oligosaccharides *in vitro* to produce interleukin 1. Spleen cells from mice given these oligosaccharides also underwent acceleration of interleukin 2 production and cytolytic T-lymphocyte differentiation. Thus, the antitumor mechanism of NACOS-6 and COS-6 was assumed to involve increased production of lymphokines including interleukins 1 and 2, sequentially, leading to the manifestation of antitumor effect through proliferation of cytolytic T-lymphocytes.

Keywords——hexa-*N*-acetylchitohexaose; chitohexaose; interleukin 1; interleukin 2; Meth-A solid tumor; growth-inhibitory effect

Introduction

Many polysaccharides were shown to exhibit growth-inhibitory effects against solid-type tumors in experimental animals.[1–5] In the preceding papers,[6,7] however, we revealed that hexa-*N*-acetylchitohexaose (NACOS-6), one of the homologs of *N*-acetylchitooligosaccharides (NACOS) consisting solely of β-1,4-linked *N*-acetyl-D-glucosamine, exhibited a significant enhancing effect on the immunological function of neutrophils in terms of an increase in the number of attracted peritoneal exudate cells in mice and the amount of active oxygen species produced by these cells. We further reported that NACOS-6 and chitohexaose (COS-6) were growth-inhibitory against sarcoma 180 and MM-46 solid tumors transplanted into mice when given by i.v. administration.[8] In this paper, we report the results of an additional antitumor assay on NACOS-6 and COS-6 using a syngeneic Meth-A solid tumor in BALB/c mice, as well as the results of an analytical study of the action mechanism of these oligosaccharides.

Materials and Methods

Chemicals——Hexa-*N*-acetylchitohexaose (NACOS-6) [*O*-β-2-acetamido-2-deoxy-D-glucopyranosyl-(1→4)-*O*-2-acetamido-2-deoxy-β-D-glucopyransoyl-(1→4)-*O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-*O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-*O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucose], penta-*N*-acetylchitopentaose (NACOS-5) [*O*-β-2-acetamido-2-deoxy-D-glucopyranosyl-(1→4)-*O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-*O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-*O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucose], chitohexaose (COS-6) [*O*-β-2-amino-2-deoxy-D-glucopyranosyl-(1→4)-*O*-2-amino-2-deoxy-β-D-glucopyranosyl-(1→4)-*O*-2-amino-2-deoxy-β-D-gluco-

pyranosyl-(1→4)-*O*-2-amino-2-deoxy-*β*-D-glucopyranosyl-(1→4)-*O*-2-deoxy-*β*-D-glucopyranosyl-(1→4)-*O*-2-deoxy-*β*-D-glucopyranosyl-(1→4)-2-amino-2-deoxy-D-glucose], and chitopentaose (COS-5) [*O*-2-amino-2-deoxy-D-glucopyranosyl-(1→4)-*O*-2-amino-2-deoxy-*β*-D-glucopyranosyl-(1→4)-*O*-2-amino-2-deoxy-*β*-D-glucopyranosyl-(1→4)-*O*-2-amino-2-deoxy-*β*-D-glucopyranosyl-(1→4)-2-amino-2-deoxy-D-glucose] were supplied by Ihara Chemical Industry, Tokyo, Japan. All oligosaccharides used in the present study were free from bacterial endotoxins as assayed by the method of Morita *et al.*[9] Each oligosaccharide was administered to mice as sterilized saline solution.

Recombinant interleukin 2 (Genzyme Co., Ltd. MA, U:S.A.) was used as a solution in RPMI-1640 medium.

Animals——Mice used in this study were male BALB/c strain, 5 to 7 weeks old, purchased from Shizuoka Experimental Animal Agriculture Corp., Shizuoka, Japan.

Tumor Cells——Meth-A tumor cells were kindly donated by Dr. K. Nitta, National Cancer Center Research Institute, Japan and were maintained in ascites form in the peritoneal cavity of male BALB/c mice.

Cell Lines——Murine IL-2-dependent cytotoxic T-lymphocyte cell line (CTLL-2, a gift from Dr. K. Kumagai) was grown in RPMI-1640 (Nissui Seiyaku Co., Ltd., Tokyo) containing 10% fetal bovine serum (abbreviated as 10% FBS PRMI-1640), 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

Antitumor Assay——Mice were transplanted s.c. with Meth-A tumor cells, 5×10^5/mouse, in the right groin. At 14 d after the tumor implantation, mice (7—10/group) were given NACOS or COS *via* the tail vein, and the tumors were extirpated and weighed on after 30 d after the tumor implantation. The tumor growth-inhibition ratio was calculated by means of the following formula:

$$\text{inhibition ratio } (\%) = 100 \times (A - B)/A$$

where A and B represent the average tumor weights of the untreated (saline-administered) group and that of the group given NACOS or COS, respectively.

Assay of Delayed-Type Hypersensitivity (DTH) Response——DTH assay using Meth-A tumor cells as the syngeneic antigen was conducted as follows. Mice were given NACOS-6, NACOS-5 or COS-6 at 14 d after transplantation of Meth-A tumor cells, 5×10^5/mouse, in the right groin. Four days later, the mice received an s.c. injection of a saline suspension of Meth-A tumor cells, 5×10^5/ml, 50 μl, as the sensitizing antigen in the left foot pad. Swelling of the foot pad was determined 24 h after injection of the tumor cells. In addition, assay of the enhancing effect of NACOS-6, NACOS-5, and COS-6 on the DTH response of normal and tumor-bearing mice using sheep red blood cells (SRBC) as the xenogeneic antigen was conducted in accordance to the description of Lagrange *et al.*,[10] as follows. Mice transplanted with Meth-A tumor cells, 5×10^5/mouse, were given i.v. administration of NACOS-6, NACOS-5 or COS-6, 10 mg/kg, followed by s.c. injection of SRBC (1×10^8 cells/50 μl) into the foot pad after 14 d. Four days later, SRBC, 1×10^8 cells/50 μl, were injected into the other foot pad, the swelling of which was determined after 24 h. Mice injected with saline instead of Meth-A tumor cells were used as the control.

Preparation of Thymus Cells——Thymus extirpated from BALB/c mice (5 to 6 weeks old) was minced by using two pieces of slide glass in Eagle's minimum essential medium (MEM: Nissui Seiyaku Co., Ltd., Tokyo), and the resultant cell suspension was centrifuged at $250 \times g$ for 5 min. The cells were further washed twice by centrifugation with MEM, and were suspended in 10% FBS RPMI-1640; the cell density was adjusted to 1×10^8/ml.

Preparation on Spleen Cells——Spleen from BALB/c mice was gently minced with two pieces of slide glass in MEM, and the resultant suspension was centrifuged at $250 \times g$ for 5 min. The cells were washed twice by centrifugation with MEM. The cells were then suspended in Tris buffer, Tris (hydroxymethyl)-aminomethane 0.17 M and ammonium chloride 0.16 M, pH 7.2, in order to allow lysis of contaminating erythrocytes. Then the cells were resuspended in fresh MEM, and the suspension was cultured in plastic Petri dishes for 2 h. The non-adherent cells were suspended in 10% FBS-added RPMI-1640 medium to obtain a suspension of 1×10^7 cells/ml. The suspension was then passed through a Nylon wool column, and the non-adsorbed cells were collected by centrifugation, and resuspended in fresh 10% FBS RPMI-1640 medium to obtain a suspension of 2×10^6 cells/ml.

Assay of Interleukin 1 (IL-1) Activity——Mouse macrophages (Mϕ) used in this study were the casein-elicited peritoneal exudate cells (PEC) obtained by i.p. administration of 2% (w/v) saline solution of casein 4 d before the collection of PEC. The content of Mϕ in PEC was more than 90% as determined by the use of May-Giemsa stain. The Mϕ (1×10^6 cells/ml) were placed in 24-well plates, and incubated at 37 °C for 48 h. The supernatant was passed through a 0.45 μm Millipore filter for sterilization, and the filtered supernatant (100 μl) was added to a suspension of thymus cells, 1×10^7/ml, and cultured at 37 °C for 48 h in 5% CO_2-air. In the last 40 h of culture, a solution of 0.5 μCi of [6-^{3}H]-thymidine (^{3}H-TdR: Amersham, U.K.) in RPMI-1640 medium, 20 μl, was added to the thymus cells. After collection of the cells by a cell harvester, the cells were assayed for incorporated radioactivity by means of a liquid scintillation counter.

Assay of Interleukin 2 (IL-2) Activity——Spleen cells used in this assay were obtained from tumor-bearing mice which were further treated with NACOS or COS at 14 d after the tumor transplantation. A suspension of spleen cells, 1×10^6 cells, in 100 μl of 10% FBS RPMI-1640 medium containing concanavalin A (Con A), 5 μl/ml, placed in a 96-well microtiter plate was cultured for 48 h in air containing 5% (v/v) CO_2. After filtration on a Millipore filter (pore size, 0.45 μm), the supernatant, 100 μl, was added to a suspension of CTLL-2 (1×10^5/ml) in 10% FBS RPMI-1640 medium, 100 μl, placed in a 96-well microtiter plate, and the cells were incubated at 37 °C for 48 h in air containing 5%

(v/v) CO_2. At 40 h before the end of culture, a solution of ^{3}H-TdR, 0.5 μCi in 20 μl, was added to the cell suspension. After the cultivation, the cells were harvested, and the incorporated radioactivity was determined by means of a liquid scintillation counter.

Assay of Sensitivity of Spleen Cells to IL-2——This was conducted by using the same spleen cells as those used in Assay of IL-2 Activity. However, the density of the starting cell suspension in 10% FBS RPMI-1640 medium was 1×10^7/ml. Aliquots of 100 μl of this suspension were placed in wells, a 10% FBS RPMI-1640 solution of mouse recombinant IL-2 (50 U/well; 100 μl) was added to the cell suspension, and the mixture was incubated for 48 h at 37 °C. At 40 h before the end of culture, ^{3}H-TdR, 0.5 μCi in 20 μl of RPMI-1640 medium, was added to the cell suspension. Determination of the incorporated radioactivity was conducted by means of a liquid scintillation counter after collection of the cells on a glass filter paper.

Assay of Cytolytic Activity of T Cells——The method was essentially the same as that described by Hashimoto and Sudo.[11] Namely, spleen T cells were obtained from Meth-A tumor-bearing mice, which were given with NACOS-6 or COS-6 at 14 d after the tumor implantation. Extirpation of the spleen was conducted 4 d after the administration of each hexaose. The T-lymphocytes prepared by the same method as described in Assay of IL-2 Activity were suspended in 10% FBS RPMI-1640 medium to give the final density of 2×10^6/ml. Incorporated radioactivity was determined by means of a liquid scintillation counter. The labeled Meth-A cells were washed twice with RPMI-1640 medium by centrifugation, and the density was adjusted to 2×10^4 ml. The cytolytic ratio of Meth-A tumor cells was assayed at the ratio of effector to target of 100 : 1, on a microtiter plate. Cytolytic ratio (%) was calculated by using the following formula:

$$\text{cytolysis } (\%) = 100 - (A/B) \times 100$$

where A and B represent experimental and normal effects, respectively.

Results

Growth-Inhibitory Effect of NACOS-6 or COS-6 on Meth-A Solid Tumor

Table I shows the growth-inhibitory ratios of Meth-A solid tumor implanted in mice after treatment with various doses of NACOS-6 , from 0.01 to 100 mg/kg. NACOS-6 at doses from 1.0 to 100 mg/kg showed an antitumor effect and the highest inhibition ratio was obtained at the dose of 10 mg/kg. Therefore, the dose of NACOS-6 for further studies was set at 10 mg/kg. COS-6 was also found to exhibit nearly the same growth inhibition ratio as that displayed by NACOS-6 at the dose of 10 mg/kg (Table II). It should be noted that no acute toxic symptom was observed in mice even at the highest dose of NACOS-6 , 100 mg/kg. NACOS-5 and COS-5, the lower homologs of NACOS-6 and COS-6, respectively, did not inhibit the tumor growth.

Release of IL-1 from Casein-Elicited Mϕ by Treatment with NACOS-6 or COS-6 in Vitro

Table III summarizes the results of the assay of IL-1 with NACOS-6 and COS-6 as the stimulants. NACOS-6 was found to enhance IL-1 production by Mϕ at the concentration of

TABLE I. Growth-Inhibitory Activity of NACOS-6 on Meth-A Solid Tumor Implanted in BALB/c Mice by Intravenous Administration

Sample	Dose (mg/kg)	Tumor weight[a] (g)	Inhibition (%)	P[b]
Control		8.8±0.5		
NACOS-6	0.01	8.6±0.8	2	NS
	0.1	7.9±0.8	10	NS
	1.0	6.5±0.8	26	<0.05
	10.0	5.1±0.5	42	<0.001
	100.0	5.3±0.7	39	<0.001

a) Mean±standard error. *b*) *Versus* the control group.

TABLE II. Growth-Inhibitory Effect of NACOS and COS on Meth-A Solid Tumor Implanted in BALB/c by Intravenous Administration

Samples	Dose (mg/kg)	Tumor weight[a] (g)	Inhibition (%)	P[b]
Control		9.5±0.4		
NACOS-6	10.0	5.3±0.8	44	<0.01
NACOS-5	10.0	8.3±0.4	12	NS
COS-6	10.0	5.6±1.0	41	<0.01
COS-5	10.0	9.6±0.3		NS

a) Mean±standard error. *b*) *Versus* the control group.

TABLE III. Release of Interleukin 1 from Macrophages Treated with NACOS or COS *in Vitro*

Samples	Dose (mg/ml)	^{3}H-TdR Incorporation[a] (DPM)	Ratio	P[b]
Control		2125.9±122.6	1.0	
NACOS-6	0.01	2210.3±167.4	1.0	NS
	0.1	8339.6±234.0	3.9	<0.001
	1.0	9403.9±415.6	4.4	<0.001
NACOS-5	1.0	4822.9±237.2	2.3	<0.001
COS-6	1.0	6069.8±134.9	2.9	<0.001
COS-5	1.0	2395.7±257.5	1.1	NS

a) Mean±standard error. *b*) *Versus* the control group.

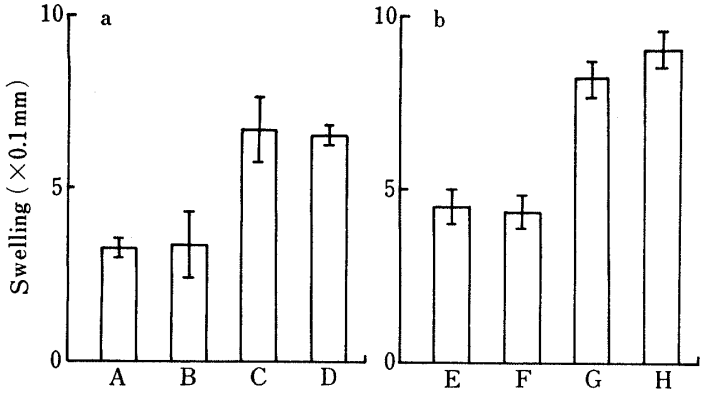

Fig. 1. Effect of NACOS and COS on DTH Response against SRBC in Tumor-Bearing Mice (a) and Normal Mice (b)

a: A, control; B, NACOS-5; C, NACOS-6; D, COS-6. b: E, control; F, NACOS-5; G, NACOS-6; H, COS-6.

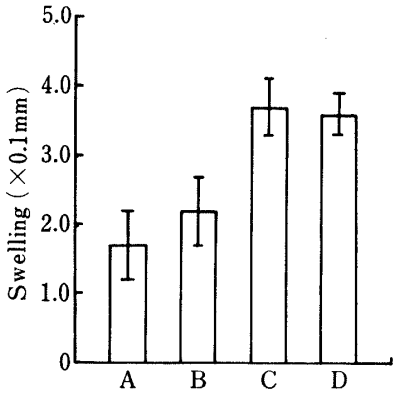

Fig. 2. Effect of NACOS and COS on DTH Response against Meth-A in Tumor-Bearing Mice

A, control; B, NACOS-5; C, NACOS-6; D, COS-6.

100 μg/ml, while COS-6 showed a lower effect than that of NACOS-6 at the concentration of 1 mg/ml. The effect of NACOS-5 was slightly less than that of NACOS-6, and COS-5 was practically inactive in this assay.

Effect of NACOS-6 and COS-6 on DTH Response to SRBC

It is generally accepted that mammals bearing tumors fall into the immunologically compromised state, in which the immunological functions of the hosts undergo significant repression. In order to analyze the action mechanisms of NACOS-6 and COS-6, therefore, it seemed to be important to obtain information concerning the accelerating effects of these oligosaccharides on the DTH response of tumor-bearing mice using the same tumor cells and sheep blood cells as the test antigens. The results of this assay, depicted in Fig. 1, indicate that, in normal mice, foot pad swelling induced by i.v. administration of NACOS-6 or COS-6 was approximately 2-fold stronger than that observed in untreated mice. However, NACOS-5 was unable to enhance this response. In a similar assay, DTH response exhibited by tumor-bearing mice given NACOS-6 and COS-6 was significantly higher than that by the untreated tumor-bearing mice. It is therefore evident that both NACOS-6 and COS-6 are able to enhance this response.

TABLE IV. Release of Interleukin 2 from Spleen Cells Tumor-Bearing Mice

Samples	Dose (mg/kg)	^{3}H-TdR Incorporation[a] (DPM)	Ratio	P[b]
Control		292.7± 81.9	1.0	
NACOS-6	10.0	794.5±110.7	2.7	<0.05
NACOS-5	10.0	279.9± 66.1	0.9	NS
COS-6	10.0	487.5± 81.7	1.7	<0.05

a) Mean±standard error. b) *Versus* the control group.

TABLE V. Effect of NACOS-6 and COS-6 on Activation of Cytotoxic T-Cells in Spleen of Tumor-Bearing Mice

Samples	Dose (mg/kg)	Cytolysis[a] (%)	Ratio	P[b]
Control		12.1±4.3	1.0	
NACOS-6	10.0	29.9±2.1	2.5	<0.05
COS-6	10.0	34.2±2.4	2.8	<0.01

a) Mean±standard error. b) *Versus* the control group.

TABLE VI. Effect of NACOS-6 and COS-6 on Response to Interleukin 2 of Spleen Cells in Tumor-Bearing Mice

Samples	^{3}H-TdR Incorporation[a] (DPM)	Ratio	P[b]
Control	106.5±13.1		
Control+IL-2 (50 U)	228.8±28.4	1.0	
NACOS-6+IL-2 (50 U)	759.5±75.4	3.3	<0.01
COS-6+IL-2 (50 U)	484.5±60.7	2.1	<0.01

a) Mean±standard error. b) *Versus* the control group.

Effect of NACOS-6 and COS-6 on DTH Response to Meth-A Tumor-Bearing Mice Using the Same Tumor Cells as Secondary Sensitizing Antigen

Figure 2 shows the DTH response of tumor-bearing mice treated with NACOS-6 or with COS-6 prior to the secondary sensitization with Meth-A tumor cells. As already pointed out, NACOS-6 and COS-6, both of which were growth-inhibitory to Meth-A solid tumor, significantly enhanced the DTH response. Summarizing the results shown in Figs. 1 and 2, therefore, it appears that NACOS-6 and COS-6 are able to enhance cellular immunity in mice in either state, normal or tumor-bearing, by accelerating the induction of IL-1.

Effect of NACOS-6 and COS-6 on Induction of IL-2 by Spleen Cells from Tumor-Bearing Mice

Although both NACOS-6 and COS-6 were unable to induce the production of IL-2 by spleen cells *in vitro* (data not shown), as shown in Table IV, NACOS-6 and COS-6 were found to enhance IL-2 production *in vivo*, approximately 2.7- and 1.7-fold, respectively. The fact that NACOS-5 did not manifest this effect suggests the dependency of this antitumor effect on the induction of IL-2.

Effect of NACOS-6 and COS-6 on Cytolytic T-Cell Activity in Spleen Cells from Mice by i.v. Administration

As summarized in Table V, enhancement of the cytolytic effect of T-cells can be observed by treatment of the tumor-bearing mice with either hexaose, *i.e.*, both NACOS-6 and COS-6 were found to elevate this effect to nearly the same extent, approximately 2.5-fold. These results indicate that administration of NACOS-6 or COS-6 to tumor-bearing mice induced the differentiation of cytolytic T-cells possessing higher killing effect.

Enhancing Effect of NACOS-6 and COS-6 on Response of Mouse Spleen Cells to IL-2

As shown in Table IV, spleen cells from mice treated with NACOS-6 or COS-6 showed a higher response to IL-2, approximately 3.3- and 2.1-fold, respectively. However, NACOS-5 did not affect the responsiveness of spleen cells to IL-2. Therefore, it appears that NACOS-6

and COS-6 are capable of enhancing not only IL-2 production but also the response of spleen cells to IL-2.

Discussion

In the present study, it was revealed that NACOS-6 and COS-6 were growth-inhibitory to Meth-A solid tumor transplanted in BALB/c mice. This confirms our previous finding,[8] *i.e.*, these hexaoses were able to inhibit the growth of sarcoma 180 and MM-46 solid tumors transplanted in mice. However, another finding obtained in the present study was that NACOS-5 and COS-5, the lower homologs of NACOS-6 and COS-6, were unable to exhibit the same effect. These results suggest that the molecular size of NACOS-6 and COS-6, corresponding to 6 *N*-acetyl-D-glucosamine and D-glucosamine residues, respectively, can be regarded as one of the essential elements for manifesting the antitumor effect.

It is well-known that lentinan, an antitumor β-glucan can enhance the production of IL-1 from Mϕ, and this effect serves as the trigger of differentiation and proliferation of tumor-killing cells such as cytolytic T-lymphocytes.[12,13] Similarly, NACOS-6 and COS-6 were able to enhance the release of IL-1, and to elevate the DTH response to both SRBC and the tumor cells in Meth-A tumor-bearing mice. However, NACOS-5 and COS-5 were unable to enhance IL-1 production or DTH response, in accordance with the result fact that NACOS-6 and COS-6 can elevate the production of IL-2 by spleen cells, but NACOS-5 did not.

The above findings led us to postulate that activation of the Mϕ might take place by direct incorporation of either NACOS-6 or COS-6, thus inducing the production of IL-1, which in turn accelerates the maturation of lymphocytes secreting IL-2, a cytokine required for differentiation of cytolytic T-lymphocytes. This is consistent with the finding of Gillis and Mizel *et al.*,[14] *i.e.*, IL-2 production from T-cells requires the activation of Mϕ, one of the major secretors of IL-1. Robb *et al.*[15] reported that IL-2 was necessary to increase the density of the specific receptor on the surface of T-cells in order to display the biological effect. Further, it was shown that the expression of IL-2 receptor requires some factor released by Mϕ, as reported by Lipkowitz *et al.*[16] and Wakasugi *et al.*[17] These results suggested that activated Mϕ or their product can induce the expression of IL-2 receptor, thus enhancing the responsiveness to IL-2 of spleen cells. Because i.v. administration of either NACOS-6 or COS-6 enhanced the IL-2 response by spleen cells *in vitro*, causing maturation of killer T-cells, it is possible that some products released from the activated Mϕ by the action of NACOS-6 or COS-6 play an important role in the expression of IL-2 receptor on T-lymphocytes. Therefore, the mechanism of the antitumor effect of NACOS-6 and COS-6 can be assumed to be acceleration of the production of and response to IL-1 and IL-2 for maturation of splenic T-lymphocytes to killer T-cells.

However, concerning the enhancing effect on production of IL-1 from Mϕ, NACOS-6 showed a greater effect than COS-6 did. These results suggest that the mechanism of antitumor activity of COS-6 is different from that of NACOS-6, and IL-1 may not be involved in the cascade process of anti-tumor activity caused by COS-6.

Another significant finding obtained in the present study is that the molecular size of NACOS-6 and COS-6, corresponding to six GlcNAc and GlcN residues, respectively, is important for manifestation of the immunopotentiating effect of these hexaoses, and that the receptors located on Mϕ corresponding to the 6 residues of *N*-acetyl-D-glucosamine or D-glucosamine might participate in key process in the immunological enhancing effect of these hexaoses, chitin, chitosan, and parasitic living organisms containing these polysaccharides. It is well-known that mouse Mϕ possess lectin-like mannose/*N*-acetyl-D-glucosamine (Man/GlcNAc) receptors, in which the size of the combining site corresponds to 3 sugar residues and the function might be the clearance of blood plasma glycoproteins.[18–20]

Furthermore, the presence of similar receptors on the surface of human monocytes has been suggested by Matheson *et al.*[21] based on their finding that activation of these cells took place on treatment with D-glucosamine. However, findings obtained in the present study allow us to presume that the receptors for NACOS-6 or COS-6 dominating the immunopotentiating effect might be considerably different from the so-called Man/GlcNAc receptor of relatively small size (up to 3 sugar residues). Confirmation of the existence of a GlcNAc receptor corresponding to 6 consecutive GlcNAc residues on the surface of mouse and human Mϕ, spleen cells, and other immunocompetent cells seems to be an interesting field for further study.

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