Carbohydrate Research, 151 (1986) 403–408
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Note

Antitumor effect of hexa-*N*-acetylchitohexaose and chitohexaose*

KO SUZUKI, TAKESHI MIKAMI, YOSHIO OKAWA, AKIO TOKORO, SHIGEO SUZUKI†, AND MASUKO SUZUKI
Department of Microbiology, Tohoku College of Pharmacy, Komatsushima 4-4-1, Sendai 983 (Japan)
(Received August 26th, 1985; accepted for publication in revised form, December 11th, 1985)

In previous reports, we revealed that *N*-acetylchito-oligosaccharides, from tetra-*N*-acetylchitotetraose to hepta-*N*-acetylchitoheptaose, display strong attracting responses to peritoneal exudate cells (PEC) in BALB/c mice, whereas chito-oligosaccharides, from chitobiose to chitohexaose, did not show this effect. The PEC attracted by hexa-*N*-acetylchitohexaose‡ (**1**) were found to generate the largest amount of active oxygen atoms in the cells in comparison with such other attractants as lower *N*-acetylchito-oligosaccharides, chito-oligosaccharides, muramyl dipeptide, and casein. Additionally, the PEC of BALB/c mice attracted by **1** were shown to kill the cells of opportunistic pathogens, *Candida albicans, Pseudomonas aeruginosa,* and *Listeria monocytogenes,* in both *in vitro* and *in vivo* systems[1–3].

As a succeeding study, we investigated the antitumor effect of **1** and chitohexaose (**2**) against an allogenic mouse tumor system, sarcoma 180 solid tumor implanted in ddY mice, in order to explore the possibility of developing oligosaccharide immunotherapeutic agents that might possess properties safer than those of the polysaccharides and/or complex carbohydrates on which extensive studies have been conducted by many workers[4,5].

Many polysaccharides of plant origin have been shown to exhibit tumor growth-inhibitory effect against experimental mouse tumors, including both allogenic and syngeneic systems[4,5]. Their mechanism of action involves enhancement of the immunological system in the host animal, especially of such tumoricidal immuno-

*Dedicated to Roger W. Jeanloz.

†Second Department of Hygienic Chemistry, Tohoku College of Pharmacy, Komatsushima 4-4-1, Sendai 983, Japan. To whom correspondence should be addressed.

‡Abbreviations: **1**; *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)-*O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucose. **2**; hexahydrochloride of *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)-*O*-2-amino-2-deoxy-β-D-glucopyranosyl-(1→4)-*O*-2-amino-2-deoxy-β-D-glucopyranosyl-(1→4)-2-amino-2-deoxy-β-D-glucose.

0008-6215/86/$ 03.50 © 1986 Elsevier Science Publishers B.V.

cytes as cytotoxic T-lymphocytes[6], macrophages[7] and natural killer cells[8].

We have earlier studied the immunopotentiating effect of polysaccharides from yeast cell-wall, namely, α-D-mannan[9,10], β-D-glucan[11], as well as chitin and chitosan[12,13], and showed that these polysaccharides display antitumor and/or antimicrobial effects.

However, no instance has yet been reported on the immunopotentiating effect of oligosaccharides obtained by partial degradation of the antitumor polysaccharides. The use of such oligosaccharides would seem advantageous because they are safer than polysaccharides as they lack antigenicity and are less prone to accumulation in the host animal.

The finding by Matheson *et al.*[14] that D-glucosamine is able to enhance natural killer-cell activity led us to an assumption that oligosaccharides consisting of D-glucosamine and/or *N*-acetyl-D-glucosamine might display positive immunostimulating effects, including tumor growth-inhibition. The fact that muramyl dipeptide, a strong B-cell mitogen developed by Chedid *et al.*[15], contains one *N*-acetyl-D-glucosamine residue also seemed to substantiate the foregoing assumption.

The preceding study[2] on the immunopotentiating effect of the series di-*N*-acetylchitobiose to hepta-*N*-acetylchitoheptaose revealed that the higher members of this series, namely tetra-*N*-acetylchitotetraose to hepta-*N*-acetylchitoheptaose were active in attracting the PEC of BALB/c male mice, when administered intraperitoneally in a dose of 50 mg/kg. The PEC were also found to retain large amounts of active oxygen atoms, and to kill viable cells of *Candida albicans*. Compound **1** exhibited the strongest effect in attracting the PEC and in inducing active oxygen atoms among all compounds studied including tetra-*N*-acetylchitotetraose to hepta-*N*-chitoheptaose, casein, and muramyl dipeptide; the last two of these were used as positive controls. Subsequently, we investigated the bactericidal assay of PEC of BALB/c mice pretreated with **1** against viable cells of *Pseudomonas aeruginosa* and *Listeria monocytogenes*, and showed that the PEC were able to kill the viable cells of either opportunistic pathogen *in vitro*. These results strongly suggested that **1** might display a tumor growth-inhibitory effect as well as an antimicrobial effect. We therefore conducted a series of antitumor assays on **1** and **2**, using an allogenic mouse tumor system *in vivo*.

Table I shows the growth-inhibitory effect of **1** and **2** on sarcoma 180 solid tumor in ddY mice using lentinan, an antitumor β-D-glucan, as the positive control. It is immediately evident that **1** significantly inhibits the growth of this allogenic solid tumor, affording a high tumor growth-inhibition ratio (85%) at the dose of 100 mg/kg/day, 3 times, consecutively, *via* the intravenous route, 7 days after tumor implantation. Unexpectedly, compound **2**, a poor attractant of PEC in BALB/c mice in comparison with **1** (as reported in the previous papers[1–3]), exhibited a high tumor growth-inhibition ratio (93%).

The reason that **1** and **2** were required in larger doses than lentinan (which gave 72% inhibition at a dose of 10 mg/kg/day, once), was attributable to the rapid

TABLE I

GROWTH-INHIBITORY EFFECT OF **1** AND **2** BY INTRAVENOUS ADMINISTRATION ON SARCOMA 180 SOLID TUMOR IMPLANTED IN ddY MALE MICE

Materials	*Dose (mg/kg)*	*Tumor weight (g)*	*Inhibition (%)*	*Regression (No. of cured/ No. of treated mice)*	*P*
None	—	3.51 ± 1.00[a]	0	0/9	
Lentinan	10 × 1	0.98 ± 0.44	72	2/7	<0.05[b]
1	100 × 3	0.53 ± 0.30	85	3/7	<0.01
	100 × 5	0.00 ± 0.00	100	8/8	<0.01
2	100 × 3	0.23 ± 0.18	93	4/8	<0.01
	100 × 5	0.00 ± 0.00	100	7/7	<0.01

[a]Averages ± standard errors. [b]Versus the control group.

clearance of **1** and **2** from the body fluid. Increae of the administration frequency of **1** and **2** to 5 times gave enhanced results, with complete regression of the solid tumor in all mice observed.

We next conducted antitumor assays with **1** and **2** against a syngeneic mouse tumor system, the MM 46 solid tumor implanted in C3H/He mice. As summarized in Table II, both **1** and **2** showed high growth-inhibitory ratios, 83 and 55%, respectively, at a dose of 100 mg/kg/day, 5 times, consecutively, *via* the intravenous route 7 days after tumor implantation. Lentinan showed a moderate inhibition ratio (58%), at a dose of 10 mg/kg, once, intravenously, when administration was started 14 days after the tumor inoculation, in accordance with the conditions used by Akiyama and Hamuro[16] to afford a high effect by this β-D-glucan.

Both **1** and **2** were not directly cytocidal to sarcoma 180 and MM 46 tumor cells *in vitro* when these tumor cells were separately treated with each hexaose in RPMI 1640 medium containing 10% (v/v) of fetal bovine serum (10% FBS-RPMI), even in high concentration (10 mg/mL, data not shown). It should also be emphasized that mice treated with **1** or **2** at a dose of 300 mg/kg, intravenously, did

TABLE II

GROWTH-INHIBITORY EFFECT OF **1** AND **2** BY INTRAVENOUS ADMINISTRATION ON MM 46 SOLID TUMOR IMPLANTED IN C3H/He MALE MICE

Materials	*Dose (mg/kg)*	*Tumor weight (g)*	*Inhibition (%)*	*Regression (No. of cured/ No. of treated mice)*	*P*
None	—	3.96 ±0.53[a]	0	0/10	—
Lentinan	10 × 1	1.65 ±0.35	58	0/8	<0.05[b]
1	100 × 5	0.68 ±0.17	83	1/9	<0.01
2	100 × 5	1.80 ±0.40	55	0/9	<0.05

[a]Averages ± standard errors. [b]Versus the control group.

not show any toxic symptoms (data not shown). These findings indicate that the antitumor effect of **1** and **2** is not by direct cytocidal action on the tumor cells, and is host-mediated, as observed with several antitumor polysaccharides[4,5,9,11]. Previous findings concerning the immunopotentiating effects of **1** using BALB/c mice[1-3] strongly substantiate this assumption. The most striking finding obtained in the present study is that two simple oligosaccharides, **1** and **2**, consisting solely of *N*-acetyl-D-glucosamine and of D-glucosamine residues, respectively, can exhibit tumor growth-inhibitory effect against both allogeneic and syngeneic solid tumors. The finding suggests that some amino sugar-containing oligosaccharides, especially those possessing non-reducing terminal amino sugar residues, might be effective as immunotherapeutic agents for both cancer and opportunistic infectious diseases. The biological significance of interactions between amino sugar residues and their receptors on immunocompetent cells has been noted by a few workers. Matheson *et al.*[14] reported the activation of natural killer activity of human peripheral blood monocytes by treatment with D-glucosamine, and Suzuki *et al.*[1] described the activation of PEC and macrophages with **1** and **2**, respectively.

Although the presence in mammals of an *N*-acetyl-D-glucosamine-binding protein was shown by Warr[17], no definite conclusion was made on the biological function of the residues of this monosaccharide, which exists in the carbohydrate moieties of many glycoproteins. Our studies[1-3] may therefore be regarded as indicative of the biological significance of *N*-acetyl-D-glucosamine residues. Because compound **2** also exhibited a significant antitumor effect, it is reasonable to consider that the receptors corresponding to oligomeric D-glucosamine residues existing on the surface of some immunocompetent cells participate in a part of the antitumor effect. Analysis of the mechanisms of the antitumor effect of **1** and **2** on the syngeneic tumor system is now progressing in our laboratories.

EXPERIMENTAL

Materials. — Compounds **1** and **2** were the same as those used in the preceding study[1] and were manufactured by Ihara Chemical Industry, Tokyo, Japan. Both **1** and **2** were homogeneous in l.c. with a column of Bio-Gel P-4 using water as the eluant. Total carbon analysis after drying *in vacuo* for 48 h at 40° over P_2O_5 showed the purities of **1** and **2** to be 98.2 and 97.9%, respectively. Compound **2** was found to be more hygroscopic than **1**. The specific rotations of **1** and **2** ($c = 1.0$, $l = 1.0$, water, at 25°) were $-15.0°$ and $+6.0°$, respectively. The former value is closely to that ($-11.4°$) reported by Capon and Foster[18] for **1**. The latter value appeared also acceptable; it is lower than that ($+14.0°$) reported by Distler and Roseman[19] for chitotetraose. The ^{1}H-n.m.r. spectrum of **1** showd a 2.7:1 ratio of *N*-acetyl and H-1 peak areas at 2.2 and 4.6–5.0 p.p.m. The ^{1}H-n.m.r. spectrum of **2** showed only a trace of *N*-acetyl-group signal. Lentinan, an antitumor β-D-glucan from the edible mushroom *Lentinus edodes* (Berk), cultivated in Japan by Chihara *et al.*[4], was obtained from Ajinomoto Co., Ltd., Tokyo, Japan.

Animals. — Male mice of ddY and C3H/He strains weighing 18 ±2 g were purchased from Shizuoka Agricultural Experimental Animal Cooperation, Hamamatsu, Japan, and were housed 5/cage in air-conditioned quarters. Food and water were provided *ad libitum*.

Tumors. — The tumor cells used were sarcoma 180 and MM 46 carcinoma, supplied by Dr. K. Nitta, National Cancer Research Institute of Japan, and Dr. T. Tachibana, Research Institute for Tuberculosis and Cancer, Tohoku University, respectively. These tumors were maintained in ascites form in ddY and C3H/He mice, respectively, as described previously[10].

Antitumor assay. — The ascites form of cells of sarcoma 180 and MM 46 tumors (1×10^6 cells) were transplanted subcutaneously into the right groin of mice of ddY and C3H/He strains, respectively. Seven days after tumor inoculation, the mice were injected with **1** or with **2** in saline (pH 7.0–7.2) from the tail vein in the dose of 100 mg/kg/day, for 3 or 5 days consecutively. The mice were killed 23 days after tumor inoculation, and the tumor was weighed. The tumor growth-inhibition ratio was calculated from the following formula:

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

where A is the average tumor weight of the control group, and B is that of the treated group. This assay used 7 to 10 mice/group.

Assay of direct cytotoxic activity of **1** *and* **2** *against sarcoma 180 and MM 46 tumor cells.* — Tumor cell suspensions, 50 μL each, 2×10^6 cells/mL in 10% FBS-RPMI 1640, and 50 μL of 10% FBS-RPMI 1640 solution of **1** or of **2** at concentrations of 0.1, 1.0, and 10 mg/mL were mixed in the wells of microplates (167008 A/S NUNC, Roskilde, Denmark), and the mixture was cultured for 24 h at 37° in a CO_2 incubator. After the addition of 100 μL of 0.2% aqueous Trypan Blue, the number of the viable tumor cells in each well was counted by using a hemocytometer.

Acute toxicity test of **1** *and* **2** *on ddY mice.* — This was conducted in accordance with the description by Nagase *et al.*[20]. Groups of 10 mice received intravenous administration of 300 mg/kg of **1** or of **2** in saline solution. Mice were then observed during the next 72 h for the number of deaths.

ACKNOWLEDGMENT

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

1 K. Suzuki, A. Tokoro, Y. Okawa, S. Suzuki, and M. Suzuki, *Chem. Pharm. Bull.*, 33 (1985) 886–888.

2 S. Suzuki, K. Suzuki, A. Tokoro, Y. Okawa, and M. Suzuki, in J, Ishigami (Ed.), *Proc. 14th Int. Congr. Chemother.*, University of Tokyo Press, Tokyo, 1985, 819–820.

3 S. SUZUKI, K. SUZUKI, A. TOKORO, Y. OKAWA, AND M. SUZUKI, in R. MUZZARELLI (Ed.), *Proc. 3rd Int. Conf. Chitin/Chitosan*, Plenum, New York, 1985, 485–492.
4 G. CHIHARA, J. HAMURO, Y. MAEDA, Y. ARAI, AND F. FUJUOKA, *Cancer Res.*, 30 (1970) 2776–2781.
5 G. CHIHARA, J. HAMURO, Y. MAEDA, Y. ARAI, AND F. FUKUOKA, *Nature*, 225 (1970) 943–944.
6 G. A. TRUITT, D. K. DENNISON, R. R. RICH, AND S. S. RICH, *J. Immunol.*, 123 (1979) 745–750.
7 C. F. NATHAN, L. H. BRUKNER, S. J. C. SILVERSTEIN, AND Z. A. CHON, *J. Exp. Med.*, 149 (1979) 84–99.
8 N. MINATO, L. REID, H. CANTOR, P. LENGYEL, AND B. R. BLOOM, *J. Exp. Med.*, 152 (1980) 124–137.
9 Y. OKAWA, Y. OKURA, H. HASHIMOTO, T. MATSUMOTO, S. SUZUKI, AND M. SUZUKI, *Carbohydr. Res.*, 108 (1982) 328–334.
10 K. HASHIMOTO, Y. OKAWA, Y. OKURA, K. SUZUKI, S. SUZUKI, AND M. SUZUKI, *J. Pharm. Dyn.*, 6 (1983) 668–676.
11 D. L. WILLIAMS, J. A. COOK, E. O. HOFFMAN, AND N. R. DILUZIO, *J. Reticuloendothel. Soc.*, 23 (1978) 479–490.
12 S. SUZUKI, Y. OKAWA, K. HASHIMOTO, S. SUZUKI, AND M. SUZUKI, in S. HIRANO AND S. TOKURA (Eds.), *Proc. 2nd Int. Conf. Chitin Chitosan*, The Japanese Society of Chitin and Chitosan, Tottori University, Tottori, 1982, pp. 210–212.
13 K. SUZUKI, Y. OKAWA, K. HASHIMOTO, S. SUZUKI, AND M. SUZUKI, *Microbiol. Immunol.*, 28 (1984) 903–912.
14 D. S. MATHESON, B. J. GREEN, AND S. J. FRIEDMAN, *J. Biol. Res. Mod.*, 3 (1984) 445–453.
15 L. CHEDID, F. AUDIBERT, P. LEFRANCIRER, J. CHOAY, AND E. LEDERER, *Proc. Natl. Acad. Sci. U.S.A.*, 73 (1976) 2472–2475.
16 Y. AKIYAMA AND J. HAMURO, *Proteins, Nucleic Acid and Enzyme*, 26 (1981) 208–224.
17 G. A. WARR, *Biochem. Biophys. Res. Commun.*, 93 (1980) 737–745.
18 B. CAPON AND R. L. FOSTER, *J. Chem. Soc., C*, (1970) 1654–1655.
19 J. J. DISTLER AND S. ROSEMAN, *Methods Carbohydr. Chem.*, (1962) 305–309.
20 T. NAGASE, T. MIKAMI, S. SUZUKI, C. SCHUERCH, AND M. SUZUKI, *Microbiol. Immunol.*, 28 (1984) 997–1007.