The Effect of Oligosaccharides on the Production of Tumor Necrosis Factor-α by Macrophage-Like Cell Line J774/JA-4

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ABSTRACT. Stimulation and modulation of tumor necrosis factor-α (TNF-α) production during treatment of the murine macrophage-like cell line J774/JA-4 with 25 oligosaccharides were studied. Direct stimulation of TNF-α production by oligosaccharides was measured with a cytotoxic assay using the L929 cell line. Twelve samples showed a significantly higher production ($P \leq 0.01$) of TNF-α than the controls. Modulation of TNF-α production by treatment with oligosaccharides, followed by stimulation with lipopolysaccharide (LPS) from *E. coli*, was examined using the L929 bioassay system. In three samples TNF-α production increased significantly, but in four samples, production was reduced significantly ($P \leq 0.01$). No samples showed modulation of growth or viability of L929 cells within the first 26 hr. The present results are useful in the application of these oligosaccharides which is potentially applicable in medical and food technology. — KEY WORDS: macrophage, oligosaccharide, TNF.

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Macrophages and tumor necrosis factor-α (TNF-α) are considered to play a pivotal role in immune and inflammatory reactions [21]. Mononuclear phagocyte system (MPS) cells are an important source of TNF-α [14, 21]. Production of TNF-α by macrophages is induced by many stimulants, including LPS and bacterial antigens [21]. On the other hand, overproduction of TNF-α participates in the pathogenesis of various diseases, such as septicemia shock syndrome, hemorrhagic shock, disseminated intravascular coagulation, tuberculosis, and AIDS [5, 21]. Though regulation of TNF-α production or activities has been studied, much remains to be resolved [3]. Recognized also as immuno-modulator substances, a numbers of mono- [9, 13, 16, 17] and poly-saccharides [8, 10–12] have demonstrated various immunological functions against immunocytes. However, the effects of oligosaccharides on the production of TNF-α by macrophages have not been elucidated [4, 6, 14]. In the present study, we screened twenty-five oligosaccharides that are useful in the modulation of TNF-α production by macrophages, using the J774/JA-4 murine macrophage-like cell line [1, 2].

The present study examined twenty-five oligosaccharides, including nine cyclodextrins (CDs) (Table 1). Nine oligosaccharides (sample Nos. 1 to 15) were kindly provided by Dr. H. Nakamura of Meiji Seika Kaisha, Ltd., Saitama, Japan. Sixteen saccharide samples (sample Nos. 16 to 31) were provided by Dr. T. Fujita of the Carbohydrate Research Laboratory of Ensuiko Sugar Refining Co., Ltd., Yokohama, Japan. All samples were diluted in an RPMI-1640 medium without serum to 10 mg/m*l* and sterilized by filtration using 0.22 μm pore filters. Ultra-pure endotoxin-free water was used for all preparations in the experiments. The stock solutions were aseptically sealed and kept at 4°C until use.

The murine macrophage-like cell line JA-4, which is a clone of J774 [1, 2], was cultured at 37°C, 5% CO_2, in a complete RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% (v/v) fetal calf serum (FCS) (IANZA), 15 mM HEPES (N-2-hydroxylpiperazine-N'-2-ethanesulphonic acid) buffered solution (pH 7.2), penicillin (100 U/m*l*) and streptomycin (100 μg/m*l*). The cells were cultured in plastic plates (Falcon #1001) for three days and seeded at 5×10^4 cells/180 μ*l*/well in 96-well tissue culture plates (Falcon #3075).

After incubation for 1 h to attach the cells to the plates, 20 μ*l* (0.95 mg/m*l* in final concentration) of each sample were added to each well and incubated for 2 hr. Then, 10 μ*l* of RPMI-1640 medium with LPS (*Escherichia coli* 011:B4, Sigma) (0.1 μg/m*l* in final concentration), or without LPS, were added. After 24 hr incubation at 37°C, 5% CO_2, the supernatant in the plates was collected and centrifuged at 2,000 R.P.M. for 10 min and then kept at − 20°C until TNF-assay. The biological activity of TNF-α was measured by a cytotoxic assay performed on L929 murine fibroblast cells using the modified method described previously [15]. The effect of the oligosaccharide samples on L929 cell growth was checked before the TNF-α-bioassay. Briefly, 100 μ*l* of L929 cell suspension at 2×10^5 cells/m*l* was seeded in 96 microwells (FALCON 3075) for 24 hr. Serially diluted cell culture supernatant, or r-hTNF-α (Dainihon Pharmaceutical Co, Ltd., Tokyo, Japan: laboratory standard in authors' laboratory), were added in triplicate to culture wells with actinomycin D (at a final concentration of 1 μg/m*l*) and incubated for 20 hr.

Supernatant in the culture wells was removed, and 100 μ*l* of the neutral red solution 1 μg/m*l* in RPMI-1640 complete medium with 10% FCS were added and incubated for 1 hr. The supernatant was aspirated carefully, and 0.25% glutaraldehyde in PBS was added and fixed for 10 min at 4°C. After washing the wells three times with cold PBS at 4°C, the cells were lysed with 100 μ*l* of basic ethanol (200 m*l* 50% ethanol/4 m*l* 5N NaOH). After gentle shaking of the plates, fluorescence was measured using Cytofluor 2300 by excitation/emission of 530/645. The results were expressed in U/m*l* by comparison of r-hTNF-α (our

laboratory standard) at 50% cell lysis. Statistical analyses were carried out using Student's *t*-test. The endotoxin content in the culture media with or without oligosaccharide samples was assayed using Limulus amaebocyte lysate tests (Wako, Tokyo, Japan) or Endotoxin Test-D (Seikagaku Kogyo, Tokyo, Japan). The level was expressed as *p*g/m*l* in the working solution.

To evaluate the direct effect of oligosaccharide on TNF-α-release by macrophages, 0.95 mg/m*l* of samples was added to the medium for the cells. Twelve of 25 samples of oligosaccharide induced release of a significantly higher level ($P \leq 0.01$) of TNF-α by JA-4 than the control. The JA-4 cells secreted 1.4 to 2.2 times the TNF-α due to stimulation by various saccharides. The same concentration of saccharides did not modify the cell growth of the L929 cells.

Examination of the modulation of TNF-α production by treatment with oligosaccharide 2 hr before stimulation by LPS in *E. coli* with the L929 bioassay system. Three samples including Inulo-oligosaccharide, polygalacturonic acid-oligosaccharide, and fructosyl-xyloside, significantly increased TNF-α release by LPS stimulation (Table 1). However, four samples, glucuronic acid-oligosaccharide, unsaturated glucuronic acid-oligosaccharides, G1-α-CD, and lactosyl-fructoside, significantly suppressed TNF-α release by the macrophages (Table 1). The endotoxin contents of the media with saccharide sample that were used in the treatment of cells varied from 0.1 to 28 *p*g/m*l* (Table 1). All culture media for JA-4 were negative in the test.

A variety of stimulants are known to induce production and release of TNF-α by macrophages [21] and macrophage cell lines [1, 2] but, the effects of oligosaccharides, including cyclodextrin, on TNF production by the cells have not been well elucidated well. Application of oligosaccharides to a host for any purpose, would seem advantageous because they are safer than polysaccharides (as they lack antigenicity) and are less prone to accumulation in the host [18].

The present study suggests that some oligosaccharides stimulated macrophages significantly to produced TNF-α

Table 1. List of samples, endotoxin contents and TNF-α production[a)]

No.	Oligosaccharides	Endotoxin contents[b)] (pg/m*l*)	Single treatment with samples[c)]	Pretreatment and challenge with LPS[d)]	Origin[e)]
Cont:	Negative-control (RPMI-1640 alone)	0.574>	28.1 ± 2.7	–	
	LPS-control (no pretreatment)	105	–	291.8 ± 43.7	
1	β-Glucano-oligosaccharide (from bukuryou)[f)]	1.19	59.8 ± 18.5*	398.1 ± 62.3*	M
2	Inulo-oligosaccharide	0.26	48.1 ± 6.5**	904.6 ± 130.1**	M
3	Polygalacuturonic Acid-oligosaccharide	0.97	33.5 ± 3	1446.6 ± 358.1**	M
4	Glucuronic Acid-oligosaccharide	2.74	26.7 ± 4.4	161.5 ± 19.2 (–**)	M
5	Mannuronic Acid-oligosaccharide	0.05	26.8 ± 2.3	185.1 ± 21.3 (–*)	M
6	Unsaturated Glucuronic Acid-oligosaccharide	6.48	36.8 ± 5.4	146 ± 7.7 (–**)	M
7	Unsaturated Mannuronic Acid-oligosaccharide	6.0	191. ± 0.8	256.2 ± 10.8 (–*)	M
8	Unsaturated Hyaluronic Acid-oligosaccharide	0.15	27.4 ± 2.6(–*)	242 ± 37	M
9	Unsaturated Pectic Acid-oligosaccharide	28.0	43.6 ± 3.6**	259.8 ± 44.6	M
10	Lactosyl Fructosid	4.8	49.7 ± 9**	112.5 ± 11 (–**)	E
11	Galactosil Fructosid	1.53	42.5 ± 1.1**	528.8 ± 174.1*	E
12	Glucosil Xylosid	0.1	43.5 ± 6.3**	615.3 ± 208.8*	E
13	Maltotriose	0.72	31.9 ± 1.7	200.4 ± 22.9*	E
14	Maltopentaose	0.96	49.1 ± 4.8**	201 ± 83.9	E
15	Fructosyl Xylosid	0.34	58 ± 8.1**	415.2 ± 25.4**	E
16	Galactosil-malto-tetolaose	0.49	26.2 ± 3.3*	206 ± 30.5*	E
17	Galactosil-malto-pentaose	0.82	48.1 ± 13.8*	231.3 ± 31.8	E
18	G1-cyclodextrin	1.06	24 ± 4.5	160.5 ± 7.2 (–**)	E
19	G1-cyclodextrn	1.62	23.2 ± 5.3	200.8 ± 15.5 (–*)	E
20	G2-cyclodextrin	0.13	23.2 ± 5.3	194 ± 34.3 (–*)	E
21	G2-cyclodextrin	0.07	62.2 ± 11.8**	305.6 ± 46.8	E
22	G2-cyclodextrin	0.24	50.8 ± 2.5**	209.3 ± 12.6 (–*)	E
23	G1,G2-cyclodextrin	6.27	47.8 ± 3.2**	306.5 ± 84.5	E
24	G1,G1-cyclodextrin	11.18	48 ± 3.3**	193.1 ± 7.7 (–*)	E
25	G2,G2-cyclodextrin	0.41	40.4 ± 4.1**	431.9 ± 68.7*	E

a) TNF level expressed as units/m*l*. Statistically higher than control: *: $P \leq 0.05$, **: $P \leq 0.01$; lower than control: (–*): $P \leq 0.05$, (–**): $P \leq 0.01$.
b) Final endotoxin concentrations in the medium for assay are expressed.
c) Cells were treated with saccharide samples alone for 26 hr.
d) Cells were pre-treated with saccharides for 2 hr and stimulated with LPS for 24 hr.
e) Companies which provided the oligosaccharides are marked as follows: M=Meiji Seika Kaisha, Ltd.; E=Ensuiko Sugar Refining Co., Ltd.
f) An herbal medicine in Japan.

by themselves; the level of TNF-α was 1.4 to 2.2 times higher than the control. Five of the eight CDs stimulated macrophages by themselves. Hishinuma *et al*. [6] reported that chitosan and α- and β-CDs augmented superoxide generation from peritoneal macrophages *in vivo* in mice. They emphasized the potential of β-CD as a possible defense stimulator. Further evaluation of these functions as they relate to oligosaccharides, which showed activity in TNF-α production, is needed. Some examples of immunological activities other than TNF-α production performed by various oligosaccharides have been cited. N-Acetyl Chitohexaose was able to induce interleukin-1 (IL-1) release from macrophages *in vitro* [20]. N-acetylchitohexaose (NACOS) generated a large amount of active oxygen atoms in mouse peritoneal cells [18]. Intracellular bactericidal activity for *Candid albicans, Pseudomonas aeruginosa* and *Listeria monocytogenes in vitro* and *in vivo* was evaluated by NACOS [7, 18].

In addition, some of the oligosaccharides in the present study showed priming activity for TNF-α production as biological response modifiers (BRM). Pretreatment with inulo-oligosaccharides, polygalacturonic acid-oligosaccharides and fructosyl-xyloside significantly increased TNF-α release by LPS stimulation. In contrast to these saccharides, the other four saccharides, glucuronic acid-oligosaccharides, unsaturated glucuronic acid-oligosaccharides, lactosyl-fructoside, and G1-α-CD, significantly suppressed TNF-α release by the macrophages. Since overproduction of TNF-α is implied in many acute and chronic disease states [14, 21], the five oligosaccharides of the present study may be considered valuable in the control of pathogenic processes relating to these conditions.

A recent report on lectin-like properties of CD14/LPS receptors in competition experiments with various saccharides [4] provided useful information to elucidate the phenomenon reported herein. In the study, saccharides competed for LPS in binding to CD14 receptors; however, more of the saccharides, except for fucoidan, could induce cytokine release. The down regulation of four oligosaccharides in TNF-α production observed in our study may be caused by competition for binding to CD14. Cavaillon *et al*. reported that Poly-galacuturonic acid competed for LPS binding to CD14, but did not induce TNF-α [4]. In the present study, Poly-galacuturonic acid-oligosaccharide showed no TNF-α induction by itself, but pretreatment with it prior to LPS stimulation of macrophages showed very good up-regulation of TNF-α.

Endotoxin is a strong stimulant for TNF-α production by macrophages [19, 22–23] having a priming and triggering effect. Dose dependent, up- and down-regulation of subsequent LPS-stimulated TNF-α production reportedly occur by the priming effect. Takasuka, and Tokunaga [19] reported pre-exposure of mouse peritoneal macrophages (PEM) to low doses (> 1 *n*g/m*l*) of LPS down-regulated TNF-α production. Recently, Zhang and Morrison [24] reported that pretreatment of mouse PEM with LPS at 0.5 to 1 *n*g/m*l* up-regulated TNF-α production, but pretreatment at 5 *n*g/m*l* down-regulated it. Pretreatment of cells with less than 0.1 *n*g/m*l* of LPS did not modify TNF-α secretion by LPS. In our preliminary examination, preincubation of JA-4 cells with 1 *n*g/m*l* of LPS for 2 hr resulted in down-regulation of TNF-α production by LPS stimulation, but pretreatment with LPS under 0.1 *n*g/m*l* did not modify TNF-α production (unpublished data). In the present study, macrophages were pre-expose to various oligosaccharide samples having a very low level (0.1 *p*g/m*l* to 28 *p*g/m*l*) of endotoxin in the medium. Based on the results of previous studied and the present investigation, we considered that the effect of endotoxin on oligosaccharides can be negligible.

Yamazaki *et al*. [22] reported inhibition of TNF-α production in mice by perilla juice. However, most vegetable juices have a priming effect on TNF-α in mice [23]. The present study suggests that oligosaccharides produced by digestion of various feed sugars in the alimentary tract or those present in the lysosomes of macrophages might functional as BRM. The biological characteristics of the oligosaccharides reported here can be applied in human and animal medicine, as well as the food industry. Further experiments on the mechanism and functional structure are needed.

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