We investigated the cytokine-inducing activities of guluronate (G3–G6) and mannuronate (M3–M6) oligomers on RAW264.7 cells with the Bio-Plex assay system. Relatively high levels of tumor necrosis factor-α (TNF-α), granulocyte colony-stimulating factor (G-CSF), monocyte chemoattractant protein-1 (MCP-1), regulated upon activation normal T cell expressed and secreted (RANTES), granulocyte macrophage (GM)-CSF, and eotaxin were induced by alginate oligomers to different extents depending on the oligomer structures, and low but significant levels of interleukin (IL)-1α, IL-1β, IL-6, IL-9, and IL-13 were also induced. Throughout all cytokines tested, M-oligomers tended to be more potent than G-oligomers in terms of cytokine induction, and this tendency was evident in differences between G3 and M3.

Key words: alginate oligomers; RAW264.7 cells; tumor necrosis factor-α (TNF-α); granulocyte colony-stimulating factor (G-CSF); cytokines

Alginate is an acidic linear polysaccharide consisting of α-1-guluronate (G) and β-1-mannuronate (M); the residues are arranged in a block structure of a homopolymer (pol yguluronate or polymannuronate) or heteropolymer (a mixed sequence of these residues), and these block structures are expressed as G-blocks, M-blocks, and MG-blocks respectively. Recent studies indicate that enzymatically depolymerized alginate oligomers show a wide variety of physiological activities, such as growth promotion of bifidobacteria, increase in shoot elongation after germination of komatsuna (Brassica rapa var. pervidis) seeds, enhancement of the growth of human endothelial cells, and keratinocytes. We found that enzymatically depolymerized alginate oligomers induced the secretion of cytotoxic cytokine from human mononuclear cells. Our further studies using highly purified oligomers of defined structure (G3–G9 and M3–M9) indicated that alginate oligomers induces TNF-α secretion from RAW264.7 cells in a structure-dependent manner. Since bulk preparation of G3–G6 and M3–M6 is easier than in the case of other, larger oligomers, the effects of these purified alginate oligomers on the secretion of various cytokines other than TNF-α from RAW264.7 cells were investigated by the Bio-Plex assay system in this study.

Phosphate-free trimer to nonamer of G (G3–G6) and of M (M3–M6) oligosaccharides were prepared by alginate lyase-digestion of PG and PM as described previously. Before use, all alginate oligomers were filtered through an endotoxin-removing filter (Zetapor Dispo filter) purchased from Wako Pure Chemical Industries (Osaka, Japan). RAW264.7 cells (mouse macrophage cell line) were obtained from the American Type Culture Collection (Rockville, MD), and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 µg/ml), and streptomycin (100 µg/ml), as described previously. Mono-layers of RAW264.7 cells in 96-well plates (2 x 10⁴ cells/well) were cultured with each alginate oligomer in the growth medium. After 24 h, the supernatant was withdrawn from each well and applied to the Bio-Plex system (Bio-Rad, Tokyo). Bio-Plex mouse cytokine assay for simultaneous quantitation of tumor necrosis factor (TNF)-α, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, and IL-17, interferon (IFN)-γ, eotaxin, keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated upon activation normal T cell expressed and secreted (RANTES), and monocyte chemotactant protein (MCP)-1 were employed according to the recommended procedures. The data were analyzed using Bio-Plex Manager™ software with 5PL curve fitting. The value of each cytokine in control conditioned medium without alginate oligomer was subtracted from the value

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Abbreviations: G, guluronate; M, mannuronate; TNF, tumor necrosis factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; IFN, interferon; RANTES, regulated upon activation normal T cell expressed and secreted; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; KC, keratinocyte-derived chemokine; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay
Fig. 1. Effects of Unsaturated Guluronate Oligomers (G3–G6) and Mannuronate Oligomers (M3–M6) with Various Degrees of Polymerization on the Production of (a) TNF-α, (b) MCP-1, (c) G-CSF, (d) RANTES, (e) GM-CSF, (f) Eotaxin, (g) IL-6, (h) IL-13, (i) IL-9, (j) IL-1β, (k) IL-1α, (l) IL-12 (p70), (m) IL-12 (p40), (n) IL-5, and (o) KC from RAW264.7 Cells.

Cells in 96-well plates (2 × 10^4 cells/well) were cultured in growth medium in the presence of each purified alginate oligomer at a concentration of 500 μg/ml at 37°C. After 24 h, the supernatant was withdrawn from each well and then subjected to Bio-Plex assay to determine each cytokine level, as described in the text. The effects of polymannuronate (PM) and polyguluronate (PG) were measured in the same way. Each value represents an average of duplicate measurements. Differences between duplicate measurements for each value were within 5%.
obtained from each alginate oligomer-treated medium as a background. The concentration of G-CSF in the culture supernatant was also determined by sandwich enzyme-linked immunosorbent assay (ELISA), similar to the assay for TNF-α detection described previously. In this ELISA, anti-mouse G-CSF monoclonal antibody (R&D Systems, Minneapolis, MN) and anti-mouse G-CSF polyclonal antibody (R&D Systems, Minneapolis, MN) were used instead of anti-TNF-α antibodies.

As shown in Fig. 1, alginate oligomers induced relatively high levels of G-CSF, MCP-1, RANTES, GM-CSF, and eotaxin secretion, in addition to TNF-α from RAW264.7 cells, in a structure-dependent manner, and lower but significant levels of IL-1β, IL-6, IL-9, and IL-13 secretion, which were evidently higher than the levels in the control conditioned medium, were also induced. Although it appears that fairly low levels of IL-5, IL-12 (p70), IL-12 (p40), and KC were detected, these levels are not significant, considering that the detection limits of these cytokines are nearly 2 pg/ml by the Bio-Prex assay system. Other cytokines including IL-2, IL-3, IL-4, IL-17, IL-10, and IFN-γ, were at undetectable levels. Since even in the control conditioned medium, MIP-1α and MIP-1β were over the quantitative level of this assay system, reliable results for these cytokines could not be obtained. Among the cytokines induced by alginate oligomers, the levels of TNF-α, MCP-1, and G-CSF were strikingly higher than others. Although many compounds possessing the ability to induce CSFs in a variety of cells are known, to our knowledge this is the first report indicating that alginate oligomers, especially mannanurane oligomers (M3–M6), are capable of inducing G-CSF. G-CSF is a hematopoietic factor that stimulates neutrophil production and release from bone marrow as well as activating mature neutrophilic function. Since neutrophils are primary effectors in host defense against invading pathogens, the use of G-CSF has been tested in the treatment of bacterial infectious diseases, and an antiviral effect of G-CSF on the hepatitis virus has even been reported. Thus the ability of alginate oligomers to induce G-CSF may be a new and important bioactive aspect for the future development of alginate oligomers as therapeutic agents. As shown in Fig. 2, an ELISA assay for G-CSF also confirmed the induction of G-CSF by alginate oligomers, and a similar pattern of structure-activity relationship was observed.

Consistent with our previous ELISA assay, no significant effects of PM or PG before enzymatic digestion were observed in terms of cytokine induction (Fig. 1). Although the exact reason for the ineffectiveness of PM and PG as cytokine inducers is still unknown, unsaturated terminal structure produced by enzymatic digestion as well as fragmentation into suitable molecular sizes may be important for the activity to induce cytokine secretion. Interestingly, mannanurane oligomers tended to be more potent than guluronate oligomers as cytokine inducers, at least based on the comparison between the oligomers (G3–G6 and M3–M6) tested. This tendency was more evidently observed in the trimers (G3 and M3), and M3 showed higher activity than G3 in the induction of all cytokines examined, suggesting that molecular conformation may be an important factor influencing the cytokine-inducing activity of alginate oligomer, as well as molecular size. Regarding this point, Otterlei et al. have reported that highly mannanurane-enriched uronic acid polymers isolated from Pseudomonas aeruginosa were the most efficient polysaccharides tested in inducing TNF-α production from human monocytes.

In conclusion, our results indicate that alginate oligomers are active in inducing multiple cytokines including G-CSF, and that their activities differ depending on structure. Structure-activity relationship profiles
suggest that molecular conformation as well as molecular size is an important factor influencing cytokine-inducing activity.

Acknowledgments

This study was partly supported by Nagasaki Prefecture collaboration of Regional Entities for Advancement of Technological Excellence and Japan Science and Technology Agency.

References


