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Effect of mannuronate content and molecular weight of alginates on intestinal immunological activity through Peyer's patch cells of C3H/HeJ mice

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ABSTRACT

We studied the effect of the mole fraction (F_M) of β-D-mannuronate residues in alginate chains and the weight-average molecular weight (M_w) of alginates on intestinal immunological activity through Peyer's patch cells of C3H/HeJ mice. Over most of the range of M_w (30,000–690,000), alginates with high mannuronate content (high-M alginates, F_M 0.69–0.86) showed immunological activity, but alginates with F_M lower than 0.31 did not. For high-M alginates with F_M = 0.78 extracted from *Laminaria japonica*, those with M_w lower than 200,000 showed the highest activity.

1. Introduction

Alginates are linear polysaccharides which consist of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues, forming three types of sequences: M-blocks (MM), G-blocks (GG) and alternating structures (MG) of various lengths and relative proportions. The mole fraction (F_M) of M residues and the block length of M and G in the sequence vary among alginates isolated from different brown seaweeds (Grasdalen, 1983; Grasdalen, Larsen, & Smidsrød, 1981; Haug, Larsen, & Smidsrød, 1974; Larsen, Salem, Sallam, Mishrikey, & Beltagy, 2003; Rioux, Turgeon, & Beaulieu, 2007). Alginates with high M content (high-M alginates) possess antitumor (Fujihara & Nagumo, 1992) and cytokine production stimulatory (Espevik, Rokstad, Kulseng, Strand, & Skjåk-Bræk, 2009; Kurachi et al., 2005; Otterlei et al., 1991, 1993) activities. Otterlei et al. (1993) found that the induction of TNF-α in monocytes by high-M alginates only takes place when the alginates' molecular weight is above 200,000. According to Espevik et al. (2009), the maximum stimulatory effect occurs within the interval $0.05 < F_M < 0.4$. Interestingly, pure mannuronan ($F_M = 1.0$) has no stimulatory effect. High-M alginates (Kurachi et al., 2005), guluronate octamers and mannuronate heptamers (Iwamoto et al., 2005) induce TNF in the macrophage cell line RAW264.7. These results suggest that both the chemical structure and molecular size of alginates affect their biological activities.

Alginates extracted from seaweed are widely used in some fields such as the food industry and medicine. Some seaweeds are used as food in many countries. Among them, *Laminaria japonica* is the most popular edible seaweed in Japan. Some studies report the various biological activities of extracts from *L. japonica* (Kim, Kim, Kim, Lee, & Lee, 2006; Liu, Yoshida, Wang, Okai, & Yamashita, 1997; Okai, Higashiokai, & Nakamura, 1993; Okai, Ishizaka, Higashi-Okai, & Yamashita, 1996; Wang, Zhang, Zhang, & Li, 2008; Xue et al., 2001; Zhao, Xue, & Li, 2008; Zvyagintseva et al., 2000). *L. japonica* contains alginate (Podkorytova, Vafina, Kovaleva, & Mikhailov, 2007; Zvyagintseva et al., 1999), but the structural characterization and biological activity of this alginate have not been described.

Alginate affects the intestinal immune system. Yamada et al. (Hong, Matsumoto, Kiyohara, & Yamada, 1998) described the effect of an herbal medicine on intestinal immunological activities through Peyer's patch cells of C3H/HeJ mice. They established a method to study these intestinal immunological activities in mice. The C3H/HeJ mice carry a mutation in the *Tlr4* gene, rendering them more resistant to endotoxin (Poltorak et al., 1998). Using these mice, the immunostimulatory effects of different alginates can be compared without extensive removal of endotoxin (LPS).

In this study, we examine the effect of F_M and weight-average molecular weight (M_w) on intestinal immunological activities *in vitro* using five alginate samples with various F_M and M_w, including two prepared from *L. japonica*.

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2. Experimental

2.1. Materials

The alginate sample (Alg-A) was provided by FMC BioPolymer AS, Norway. The sample has been produced as a hot water extract from *Durvillea antarctica*, followed by acid precipitation, neutralization with sodium carbonate before drying and milling. The sample was further purified by dissolving in 0.2% NaCl aqueous solution, precipitating with ethanol, washing with ethanol and ether, and drying.

Alg-B was extracted from *L. japonica* (provided by the R&D Division, Kenko Mayonnaise Co., Ltd., Tokyo, Japan). The seaweed swollen in 60 °C water for 30 min was ground to 2 mm particles and heated in 90 °C water for 90 min. The suspension was centrifuged and the resultant supernatant filtered with a 45 μm filter, concentrated and then freeze-thawed. The solution was centrifuged and filtered with a 5 μm filter. Alginate was precipitated in the supernatant by adding ethanol to a final concentration of 37.5%, washed with ethanol and ether, and dried.

Alg-C was extracted from *L. japonica* purchased from Nakano Bussan Co. Ltd. (Sakai, Japan). The extraction of the alginate was done according to a previously reported method (Haug, 1964; Larsen, 1978). The dried seaweed was ground and passed through a 2 mm-mesh. The seaweed material (15 g) was swollen in 75 mL of 5% formaldehyde and suspended in 750 mL of 0.2 M HCl. The suspension was filtered, and the residue was washed with distilled water and suspended in 1.5 L of 0.02 N NaOH while keeping the pH above 7. After filtering the suspension, NaCl was added to the supernatant to a final concentration of 1%, and alginate was precipitated by adding an equal volume of ethanol. The precipitant was washed with ethanol and ether, and dried at room temperature.

Alg-D is an alginate extracted from *Laminaria hyperborea* stipes (SF200, obtained from FMC BioPolymer AS, Norway).

Alg-E was extracted from the *L. hyperborea* outer cortex as described by Haug (1964) and Martinsen, Skjåk-Bræk, and Smidsrød (1989). This sample can be characterized as alginate with high G content (high-G alginate).

2.2. Preparative gel permeation chromatography

The alginate sample (300 mg–1 g) was dissolved in 1.3 L of 0.05 M Na_2SO_4 solution (pH 6) and filtered with a 0.22 μm filter before injection. The sample concentration was adjusted to make the relative viscosity of the solution less than 1.4. The 1.3 L alginate solution was injected onto a Sephacryl S-500 column (20 cm i.d. × 77 cm, General Electric Company, CT) and eluted with 0.05 M Na_2SO_4 solution (pH 6) at a flow rate of 60 mL/min. Polysaccharide was detected by the phenol sulphuric acid method, and a single peak was divided into 8, 9 or 10 fractions (1.2 L or 1.8 L each). Each fraction was concentrated, dialyzed and freeze-dried. We labeled each fraction according to its name and fraction number, for example, Alg-B2 is fraction 2 of Alg-B.

2.3. Nuclear magnetic resonance (NMR) spectroscopy

We measured the proton spectra at 90 °C using a JNM-AL400 spectrometer (JEOL Ltd., Tokyo, Japan). Before NMR measurement, we reduced the viscosity of the high-molecular weight alginates by mild acid hydrolysis according to the method of Ertesvag and Skjåk-Bræk (1999). The alginate hydrolyzate was neutralized, freeze-dried and dissolved in D_2O. Five microliters of 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt was used as the internal standard for the chemical shift. 20 μL of 0.3 M triethylene tetra-amine hexa-acetate (TTHA) was added to chelate the remaining Ca^{2+}.

2.4. Size exclusion chromatography—multiangle laser light scattering (SEC-MALLS)

We made SEC-MALLS measurements on an HPLC system equipped with three straight connected columns: TSKgel G6000PWXL, G5000PWXL, and G4000PWXL (7.5 mm i.d. × 600 mm, Tosoh Corporation, Tokyo, Japan). The column outlet was connected to a Dawn Heleos-II multiangle laser light scattering photometer (Wyatt Technology Corp., CA) (λ = 658 nm) which was connected to an Optilab rEX differential refractometer (Wyatt Technology Corp., CA). The mobile phase was 0.05 M Na_2SO_4/0.01 M EDTA (pH 6). The flow rate was 0.7 mL/min. The injection volume was 100 μL, and the sample concentration was 0.2 or 0.4% for appropriate light scattering intensity. The sample solution was filtered with a 0.22 μm filter before injection. The value of the refractive index increment ($\delta n/\delta c$) for the alginate was 0.150 mL/g, according to a previous report (Vold, Kristiansen, & Christensen, 2006). Data from the light scattering and the differential refractometers were collected and processed using Astra (v. 5.3.4.14) software (Wyatt Technology Corp., CA).

2.5. Mice

Specific pathogen-free C3H/HeJ mice (female, 8 weeks old) were purchased from CLEA Japan Inc. (Tokyo, Japan) and were kept at 23 ± 2 °C. They had free access to standard laboratory chow (CE-2, from CLEA Japan Inc.) and water.

2.6. Preparation of Peyer's patch cell suspension and cell culture

The mice were sacrificed by cervical dislocation and their small intestines were exposed on sheets of clean paper. The Peyer's patches were carefully dissected out with scissors from the wall of the small intestine, and placed in RPMI 1640 medium (GIBCO, Invitrogen Corporation, CA) with 5% fetal bovine serum (FBS, Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), 0.0004% 2-mercaptoethanol (Nacalai Tesque, Inc., Kyoto, Japan) and 1% antibiotics (containing penicillin, streptomycin and amphotericin B purchased from Nacalai Tesque, Inc.). The Peyer's patch cells were dispersed by tapping gently with a rubber rod on a 70 μm and then a 40 μm cell strainer. The cells precipitated by centrifugation were washed with PBS buffer and resuspended in RPMI 1640 medium. The viable cells stained with 0.4% trypan blue (Invitrogen Corporation, CA) were counted and suspended at a density of 2.0×10^6 cells/mL in RPMI 1640 medium. 200 μL of the cell suspension, 0.3% alginate solution (filtered with a 0.22 μm membrane filter) and sterile water were dispensed in a Petri dish and cultured for 5 days at 37 °C in a humidified atmosphere of 5% CO_2–95% air. The resultant culture supernatants were used for stimulation of bone marrow cells. Water was used as a control instead of adding 0.3% alginate solution.

2.7. Measurement of immunological activity using bone marrow cells

First, we checked that there were no effects from adding alginate directly to bone marrow cells.

Bone marrow cell proliferating activity of the Peyer's patch cell suspension was measured by a previously reported method (Hong et al., 1998). Bone marrow cells were obtained from the femora of C3H/HeJ mice. The mice were sacrificed by cervical dislocation, the femora were excised, and the bone marrow cells were flushed out using a syringe with a 23-gauge needle and suspended in RPMI 1640 medium. The cells were dispersed by tapping on a 70 μm and then a 40 μm cell strainer. The cell suspension was centrifuged, and the cells were washed with PBS buffer and

resuspended in RPMI 1640 medium at three different cell concentrations; 2.3×10^5, 1.5×10^5 and 0.4×10^5 cells/mL in Exp. 1, 9.0×10^5, 4.6×10^5 and 1.8×10^5 cells/mL in Exp. 2, and 8.0×10^5, 3.2×10^5 and 1.7×10^5 cells/mL in Exp. 3. 100 μL of the bone marrow cell suspension was dispensed into a 96-well plate, and 50 μL of normal horse serum and 50 μL of culture supernatant of Peyer's patch cells were added to each well of the plate. The cells were cultured for 6 days at 37 °C in a humidified atmosphere of 5% CO_2–95% air. Proliferation of bone marrow cells was measured by Alamar Blue reduction assay (Page, Page, & Noel, 1993). At 5 h before the culture was finished, 20 μL of Alamar Blue solution was added to each well, and the cells were successively cultured. The fluorescence intensity was measured with Fluoroskan Ascent FL (Thermo Fisher Scientific Inc., MA) at an excitation wavelength of 544 nm and emission wavelength of 590 nm. The statistical significance of differences between samples and control were tested by the Tukey–Kramer test.

3. Results

3.1. Purification and fractionation of sodium alginate

Alg-B and Alg-C were prepared from *L. japonica*. The yields of the alginates were 4.2 and 23.1%.

Fig. 1 shows the anomeric region of the ^{1}H NMR spectra of five alginates including Alg-B and Alg-C. The signals were assigned by a method previously reported (Grasdalen, 1983). Alg-A, Alg-B and Alg-C show a large signal at around 4.65 ppm which is from anomeric protons (H-1) of M residues. These are typical spectra for high-M alginates. Alg-D and Alg-E show large signals at 5.08 ppm and 4.45 ppm which are from H-1 and H-5 of G residues, respectively. These are typical spectra for alginates with high G content (high-G alginates). We obtained both F_M and diad frequencies (F_{MM}, F_{GG}, $F_{GM} = F_{MG}$) through integration of these signals by a previously reported method (Grasdalen, 1983). The results are given in Table 1, along with the M_w of the samples determined by SEC-MALLS measurements. The F_Ms of Alg-B and Alg-C were estimated to be 0.78 and 0.69, respectively, confirming that the alginates extracted from *L. japonica* are indeed high-M alginates.

All alginate samples were converted into sodium alginates, and fractionated by preparative GPC to obtain fractions covering a wide range of molecular weights, but with narrow molecular weight distributions (low polydispersity), and with higher purity. Fig. 2 shows the GPC chromatogram of Alg-B. The single peak is divided into nine fractions to obtain the samples Alg-B2, Alg-B5 and Alg-B8. In

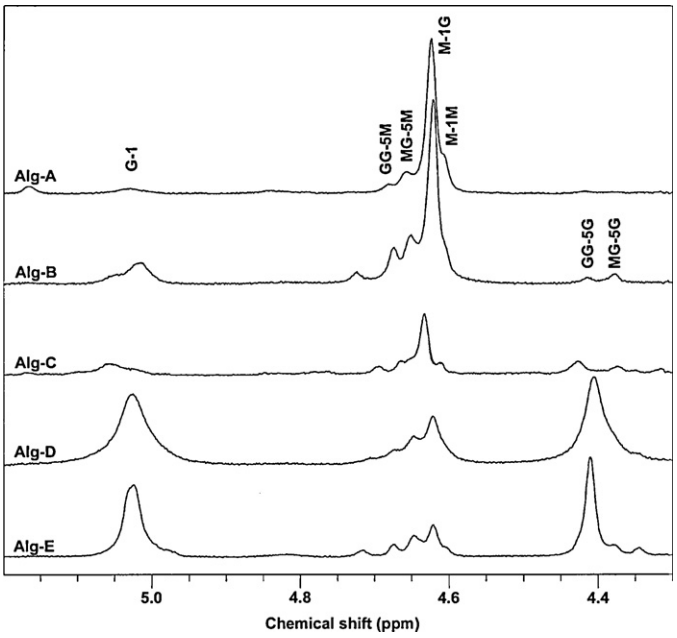

Fig. 1. Anomeric region of ^{1}H NMR spectra of the five alginates. The spectra were measured on a 400 MHz NMR spectrometer in 90 °C D_2O.

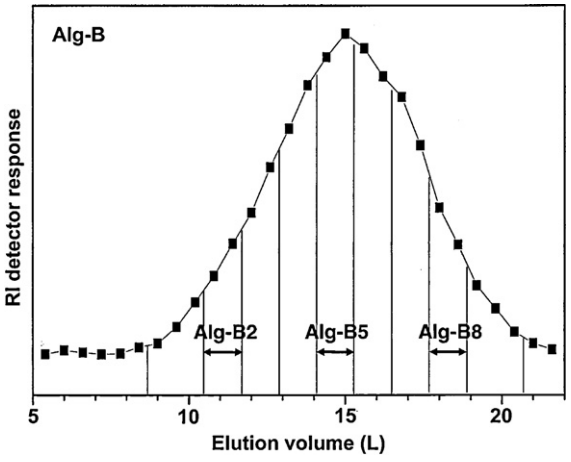

Fig. 2. Preparative GPC of the alginate sample Alg-B on a Sephacryl S-500 column.

Table 1
Mole fraction of β-D-mannuronate (F_M), weight-average molecular weight (M_w) and polydispersity index (M_w/M_n) of the alginate samples used in this study.

Sample	F_M	Mole fraction of diad sequence			M_w ($\times 10^3$)	M_w/M_n
		F_{MM}	$F_{MG,GM}$	F_{GG}		
Alg-A	0.86	0.72	0.13	0.01	130	2.20
Alg-A2	0.92	0.83	0.08	0.00	243	1.09
Alg-B	0.78	0.61	0.17	0.05	182	1.45
Alg-B2	0.80	0.66	0.15	0.05	247	1.13
Alg-B5	0.78	0.62	0.16	0.06	115	1.07
Alg-B8	0.76	0.59	0.17	0.07	30	1.13
Alg-C	0.69	0.52	0.17	0.14	507	1.16
Alg-C4	0.71	0.58	0.13	0.16	243	1.12
Alg-D	0.31	0.18	0.13	0.56	n.d	n.d.
Alg-Dl	n.d.	n.d.	n.d.	n.d.	690	1.20
Alg-D4	0.29	0.20	0.08	0.63	233	1.12
Alg-D7	n.d	n.d	n.d	n.d	48	1.13
Alg-E	0.29	0.17	0.12	0.59	370	1.34
Alg-E4	0.29	0.18	0.11	0.59	257	1.04

n.d.: not determined.

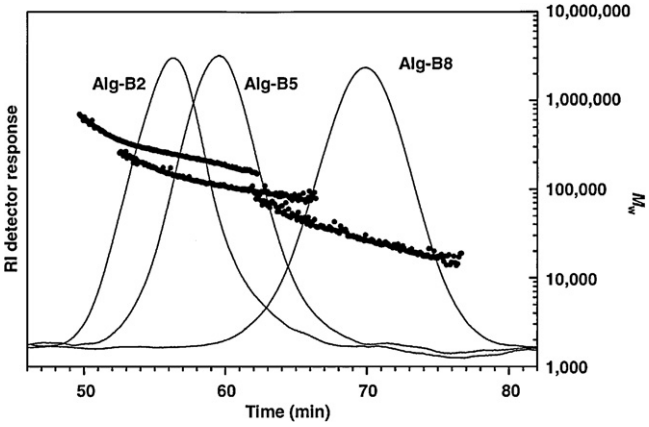

Fig. 3. SEC-MALLS chromatogram of the fraction samples Alg-B2, Alg-B5 and Alg-B8.

the same way, Alg-A, Alg-C, Alg-D and Alg-E were fractionated by GPC, and each peak on their chromatograms was divided into 9, 8, 10 and 10 fractions according to its width. The molecular weight distributions of the fractions were determined by SEC-MALLS measurements. Fig. 3 shows the SEC-MALLS chromatogram of the Alg-B fraction samples. Based on the chromatographic data, the fractions were found to have narrow molecular weight distributions ($M_w/M_n < 1.2$). The data (M_w and M_w/M_n) are also summarized in Table 1. The F_M values of the fraction samples, calculated from their ^{1}H NMR spectra, are given in Table 1. For all of the alginates, F_M had a tendency to decrease slightly with decreasing molecular weight. In the case of Alg-B, whose F_M was 0.78 before fractionation, the F_M after fractionation was 0.80 for Alg-B2 (M_w = 247,000), 0.78 for Alg-B5 (M_w = 115,000) and 0.76 for Alg-B8 (M_w = 30,000).

3.2. Intestinal immunological activity

Peyer's patch cells of C3H/HeJ mice were cultured in the presence of alginates having various F_M and M_w for direct stimulation for 5 days *in vitro*. The resultant cell-free supernatant of the Peyer's patch cell medium was used to stimulate the bone marrow cells. Fig. 4 shows the bone marrow cell proliferating activity stimulated by the Peyer's patch cell medium cultured with alginates. The first experiment (Exp. 1) shows the effect of F_M on the activity. The samples used in Exp. 1 had similar molecular weight (M_w = 243,000–257,000). Next, we studied the effects of the molecular weight (M_w) of high-M alginates (Exp. 2) and high-G alginates (Exp. 3). Alg-B was used as the reference material in all the experiments because it showed high activity in the preliminary test (data not shown). In Exp. 1, when 200 μg/mL of Alg-B was used for stimulation of Peyer's patch cells, the initial number of bone marrow cells (1.5×10^5 cells/mL) increased 25 times (3.9×10^6 cells/mL), a much greater increase than the control (1.1×10^6 cells/mL, seven times the initial cell number) in which Peyer's patch cells were incubated with water only. This result indicates that direct stimulation of Peyer's patch cells by alginate secretes immunostimulating substances such as cytokines which can induce the proliferation of bone marrow cells. As shown in Exp. 1, bone marrow cells proliferate well in the presence of Alg-A2 or Alg-C4 but much less well in the presence of high-G alginate (Alg-E4). Alg-A2 stimulated proliferation to some extent, but less than Alg-B.

Next, we studied the effect of molecular weight on the stimulatory properties of Alg-B fractions (Exp. 2). Alg-B5 and Alg-B8 more than doubled bone marrow cell proliferation compared to the control and demonstrated dose dependency. Exp. 3 shows the effect of the molecular weight of high-G alginates on stimulatory activity using Alg-D fraction samples. Bone marrow cell proliferation was not stimulated by any of the fractions Alg-D1, Alg-D4 or Alg-D7. We conclude that high-G alginates (F_M = 0.31) whose M_w ranges from 48,000 to 690,000 do not stimulate proliferation.

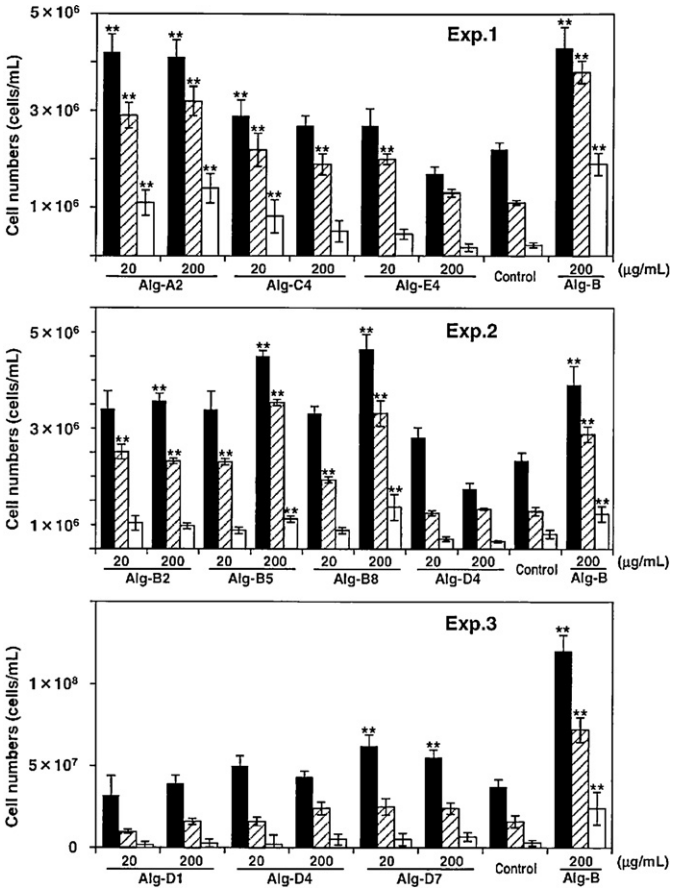

Fig. 4. Effects of F_M and M_w of alginates on immunomodulating activity. Bone marrow cells were stimulated by the culture medium supernatant of Peyer's patch cells cultured with 20 or 200 μg/mL alginate. The initial numbers of bone marrow cells were 2.3×10^5 cells/mL (■), 1.5×10^5 cells/mL (▨) and 0.4×10^5 cells/mL (□) in Exp. 1; 9.0×10^5 cells/mL (■), 4.6×10^5 cells/mL (▨) and 1.8×10^5 cells/mL (□) in Exp. 2; 8.0×10^5 cells/mL (■), 3.2×10^5 cells/mL (▨) and 1.7×10^5 cells/mL (□) in Exp. 3, respectively. The double asterisk denotes statistical significance of differences to control ($p < 0.01$, Tukey–Kramer test, in each group of initial cell numbers).

In Fig. 5, the effects of F_M on proliferation are shown for samples with approximately the same molecular weight (M_w = 233,000–257,000). The high-M alginates ($F_M \geq 0.78$) stimulated proliferation, while for the high-G alginates with $F_M < 0.31$ stimulation was marginal. This figure also shows that unfractionated Alg-B (F_M = 0.78) has intestinal immunological activity through Peyer's patch cells, whereas unfractionated Alg-C (F_M = 0.69) has low activity. Optimum activity occurs in the range F_M = 0.78–0.90 (recall that pure mannuronan lacks immunostimulatory ability) (Espevik et al., 2009).

Fig. 6 shows the effect of the molecular weight (M_w) of high-M and high-G alginates on immunostimulatory activity. For high-M alginate Alg-B (F_M = 0.78), the alginates with M_w = 30,000 and 115,000 show significantly higher activity than the one with M_w = 247,000. The high-G alginate Alg-D (F_M = 0.31) whose M_w ranges from 48,000 to 690,000 shows little or no activity.

4. Discussion

We found that high-M alginates have the ability to stimulate intestinal immunological activity through Peyer's patch cells. The cytokines secreted in culture medium by Peyer's patch cells incubated with alginates could affect the proliferation of bone marrow

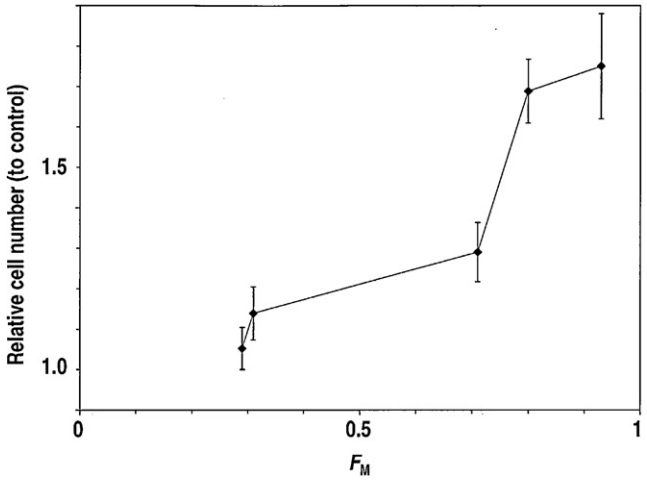

Fig. 5. Effects of F_M of alginates on the immunomodulating activity at similar M_w (ca. 250,000). Bone marrow cells were stimulated by the culture medium supernatant of Peyer's patch cells cultured with 200 μg/mL alginate. The curve shows the results for similar cell concentrations (2×10^5–5×10^5 cells/mL).

cells. To understand the secretion of these cytokines, the expression of mRNA related to immunological activity in the cells should be measured. According to a previous report (Espevik et al., 2009), TLR-4 seems to be the predominant signaling receptor for high-M alginates in murine macrophages. However, TLR-4 is not the receptor for immunological activity through Peyer's patch cells observed in this study, because the mice used here are defective in the *Tlr4* gene.

We prepared two alginate samples from *L. japonica*, Alg-B and Alg-C, and found that their F_Ms were 0.78 and 0.69, respectively. These values were higher than those of other brown seaweeds such as *L. hyperborea*, *Laminaria digitata*, *Ascophyllum nodosum* and *Macrocystis pyrifera*, which are mainly used in industry as a source of alginate. Their F_M values are reported to be somewhere between 0.25 and 0.57 (Grasdalen, Larsen, & Smidsrød, 1977, 1979; Haug et al., 1974; Kristiansen et al., 2009; Penman & Sanderson, 1972; Sandford & Baird, 1983; Thu et al., 1996), 0.58–0.62 (Grasdalen et al., 1977, 1979; Haug et al., 1974; Sandford & Baird, 1983), 0.46–0.66 (Grasdalen, Larsen, & Smidsrød, 1979; Haug et al., 1974; Penman & Sanderson, 1972; Rioux et al., 2007; Sandford & Baird, 1983) and 0.30–0.61 (Gomez, Lambrecht, Lozano, Rinaudo, & Villar, 2009; Penman & Sanderson, 1972; Sandford & Baird, 1983), respectively (some of the values were originally M/G ratios). We also found that the F_M value of the genus *Sargassum* is somewhere between 0.33 and 0.45 (Davis, Ramirez, Mucci, & Larsen, 2004; Fujihara & Nagumo, 1992; Larsen et al., 2003) and that of *Ecklonia kurome* is 0.68 (Fujihara & Nagumo, 1992). F_M varies not only with seaweeds, but also with factors such as extraction method, tissue, age and growing environment. (For example, the F_M of fruiting bodies of *A. nodosum* is 0.85; Otterlei et al., 1993.) We found that the alginate extracted from *L. japonica* by water had one of the highest F_M values among the seaweeds. Although high-M alginate with F_M higher than 0.80 can be prepared from the bacteria *Azotobacter vinelandii* (F_M = 0.81–0.89) (Grasdalen et al., 1979) and *Pseudomonas aeruginosa* (F_M = 0.94) (Jahr et al., 1997), this study suggests that *L. japonica* is a good source of high-M alginate from seaweed. The most valuable application of alginate might be food with beneficial effects on intestinal immune function, considering that *L. japonica* is traditionally eaten.

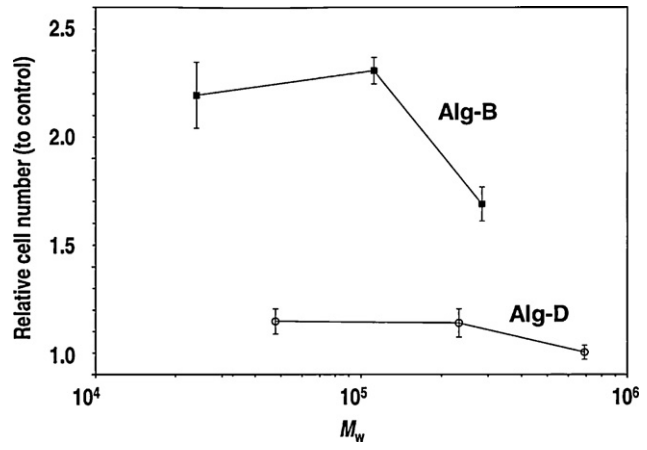

Fig. 6. Effects of M_w of alginates on immunomodulating activity for high-M alginate (Alg-B, F_M = 0.78) and high-G alginate (Alg-D, F_M = 0.31). Bone marrow cells were stimulated by the culture medium supernatant of Peyer's patch cells cultured with 200 μg/mL alginate. The curve shows the results for similar cell concentrations (2×10^5–5×10^5 cells/mL).

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