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Note

An influence of the structure of alginate on the chemotactic activity of macrophages and the antitumor activity

Michio Fujihara * and Terukazu Nagumo

Department of Biophysics, School of Hygienic Sciences, Kitasato University, Sagamihara-shi, Kanagawa 228 (Japan)

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We previously reported that sodium alginate from *Sargassum fulvellum* showed a considerable antitumor activity against various murine tumors, such as Sarcoma-180 (solid and ascitic types), Ehrlich ascites carcinoma and IMC carcinoma[1,2]. In addition to the results, we also demonstrated[2] that the alginate had the ability to enhance cytostatic and cytolytic activities of macrophages, and thus the antitumor effect may partly be caused by the activation of macrophages. Furthermore, we examined the antitumor activity of alginates from several kinds of seaweeds, and reported[3] that the antitumor activity of alginates depend on the composition of the homopolymer of 1 → 4-linked β-D-mannuronic acid (MM-block) and α-L-guluronic acid (GG-block) in alginates, i.e., alginates with a higher content of MM-block showed higher antitumor activity than those with a lower content. Recently, Otterlei et al.[4] examined alginates and their components for the ability to stimulate human monocytes to produce tumor necrosis factor-α, interleukin-6, and interleukin-1 and found that alginates stimulated the monocytes to produce all of the three cytokines at high levels. These workers also demonstrated that the mannuronic acid residues are the active cytokine inducers in alginates, which suggests that the composition of MM- or GG-blocks in alginates may also be closely correlated with the activities of macrophages. In the present paper, we describe the effect of alginates with different MM- or GG-block compositions on the chemotactic activity of macrophages, and also the influence of Ca^{2+} on the activity and the antitumor activity in relation to the structure of alginates.

RESULTS AND DISCUSSION

Alginates.—Among the alginates tested, Mm-AlgNa had the highest content of GG-block, indicating that this alginate was the only guluronate-rich one. The other

* Corresponding author.

TABLE I

Properties and antitumor activity of alginates and chemotaxis of macrophages from normal mouse treated with the polysaccharides

Alginate	Mol wt ($\times 10^5$)	MM GG^{-1}	Antitumor activity (ILS%)			Chemotaxis [a] (%)		
			Dose (mg kg^{-1})			Dose (mg kg^{-1})		
			50	25	6.3	50	25	6.3
Control						100 ± 21	100 ± 29	100 ± 10
Ek-AlgNa	2.36	3.01	162	154	77	129 ± 35	114 ± 35	93 ± 10
Ln-AlgNa	2.52	2.36	117	58	50	95 ± 5	109 ± 17	107 ± 6
La-AlgNa	2.25	2.20	31	46	38	35 ± 5	82 ± 6	109 ± 12
Sf-AlgNa	2.26	1.16	38	8	−15	27 ± 5	45 ± 20	94 ± 10
Mm-AlgNa	2.34	0.73	67	20	0	23 ± 8	38 ± 3	110 ± 19

[a] The ratio of the chemotaxis to the control (100%).

alginates were mannuronate-rich, and especially Ek-AlgNa had the highest content of MM-blocks among them (Table I).

Effect of alginates on chemotactic activities of macrophages.—The effects of alginates on the chemotaxis of macrophages from a normal mouse are shown in Table I. The mean number of migrated macrophages obtained from the mouse treated with a physiological saline solution alone as the control was 66 ± 13 in one field of the microscopic view in the experiments using three different doses. This value was used as a standard (100%). Both Ek-AlgNa and Ln-AlgNa had no effect on the chemotaxis of macrophages at any of the doses tested. Although La-AlgNa, Sf-AlgNa, and Mm-AlgNa also showed no effect at a dose of 6.25 mg kg^{-1}, they showed a remarkable effect on the chemotactic activity of macrophages at doses of 25 and 50 mg kg^{-1}. As previously described[2], we report that the antitumor activity of alginates was partly caused by the enhancement of the phagocytic activity of macrophages, and also[3] that the alginates with a higher MM-block content showed higher antitumor activity (Table I). Otterlei et al.[4] found that alginates with a high MM-block content stimulate human monocytes to produce cytokines. Therefore, we expected that the alginates with a higher MM-block content may stimulate the chemotactic activity of macrophages. However, contrary to our expectation, it was found in the present study that the alginates with higher MM-block content did not enhance the chemotactic activity and that the alginates with higher GG-block content strongly depressed the chemotactic activity of macrophages.

These results suggest that the negligible or very small antitumor activity of the alginates with higher GG-block content (Table I) may be caused by depression of the chemotaxis of macrophages. However, Mm-AlgNa showed a moderate degree of antitumor activity at a dose of 50 mg kg^{-1}, although the alginate had a high GG-block content. The reason for this behavior remains to be clarified. In order to clarify the phenomenon, studies on the antitumor activity of alginates with high GG-block content are now in progress.

TABLE II

Influence of Ca^{2+} in alginate on the chemotaxis of macrophages

Alginate (3.2 mg mL^{-1})	Ca^{2+} added (mM)	Chemotaxis [a] (%)	Ca^{2+} taken up into alginate ($\mu g\ mg^{-1}$)
Control 1	0	100±15	
Control 2	3	103±13	
Sf-AlgNa	0	19± 3	0
Sf-AlgNa	1	23±6	11.0±1.1
Sf-AlgNa	3	73± 8	37.8±4.5
Ek-AlgNa	3	93±13	30.7±2.7

[a] The ratio of the chemotaxis to control (100%).

Effects of Ca^{2+}.—It is well known that the conformation of D-mannuronan is extremely different from that of L-guluronan; the former is twisted, whereas the latter is buckled[5] and forms a so-called 'egg-box model'[6] in the presence of Ca^{2+}. Therefore, we hypothesized that the conformation of alginates, which is closely related to their MM- or GG-block content, may correlate with depression of the chemotactic activity of macrophages. In order to investigate this, we examined changes in the chemotaxis of macrophages when the alginate solution containing Ca^{2+} was injected i.p. into mice. Sf-AlgNa with a high GG-block content was used as the sample. The results are shown in Table II. A physiological saline solution containing calcium chloride alone had no effect on the chemotaxis of macrophages, whereas an alginate solution containing a greater amount of calcium chloride induced macrophages with a higher activity than that containing no Ca^{2+}. These results suggested that the alginate with lower MM-block content may also show high antitumor activity if Ca^{2+} was added to the polysaccharide solution. In order to ascertain the validity of this observation, the activity of Sf-AlgNa was examined for a polysaccharide solution with and without added Ca^{2}. As shown in Table III, although negligible or very low antitumor activities were shown at any of the doses tested for the alginate without Ca^{2+}, a significant activity was observed at a dose of 25 mg kg^{-1}, and remarkable activities were observed at a dose of 50 mg kg^{-1} of the alginate with 3 mM Ca^{2+} present.

In order to examine the changes in the structure of the above alginate in the presence of Ca^{2+}, the CD spectrum of the alginate was measured. Compared with the spectrum of native alginate, as the amount of the calcium added increased, the negative molar polarity (Fig. 1.) decreased. These CD spectral changes agreed with those reported by Grant et al.[6] and Morris et al.[7,8]. On the whole, when 3 mM Ca^{2+} was added to the alginate with a high GG-block content, the CD spectrum of the alginate approximated to that of the alginate with a high MM-block content (poly-D-mannuronate) shown by Morris et al.[8]. These results collectively suggest that a conformation of the alginate with a high MM-block content may be required for expression of the normal chemotactic activity of alginate-induced macrophages, and for the development of the antitumor activity of the polymer.

TABLE III

Effects of Ca^{2+} on the antitumor activity of sodium alginate

Sample	Ca^{2+} (mM)	Dose [a] (mg kg^{-1} day^{-1})	MSD [b]	ILS (%)	60-day survivors
None	0		15		0/7
	3		15		0/7
Zymosan	0	25	26	73	0/7
Sf-AlgNa	0	25	14	−7	0/7
	1	25	18	20	0/7
	3	25	40	167	0/7
	0	50	17	13	0/7
	1	50	30	100	0/7
	3	50	34	127	2/7

[a] Samples were injected on days 1–5 and 7–10 in the doses indicated. [b] The day on which the fourth mouse died was used as MSD (mean survival days).

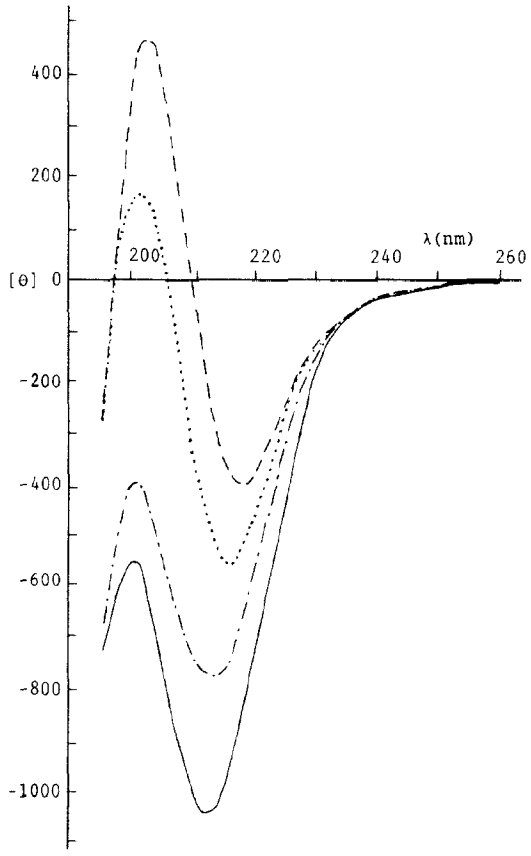

Fig. 1. CD spectra of sodium alginate (0.1%) in aqueous solution containing O mM (———), 0.5 mM (·–·–·), 1 mM (·····), and 3 mM (- - - - -) Ca^{2+}.

EXPERIMENTAL

Materials.—The brown seaweeds used in the present study were as follows: *Ecklonia kurome* (Ek), *Sargassum fulvellum* (Sf), and *Myagropsis myagroides* (Mm). These were collected on the seashore of the Sea of Japan during December, 1981 (Ek and Sf) and 1982 (Mm); *Laminaria angsutata* var. *longissima* (La) was collected on the seashore of Nemuro in May, 1982, and *Lessonia nigrescens* (Ln) was imported from Chile in 1982. They were stored at ca. −20 to −25°C until used.

Preparation methods of alginate.—Alginates were prepared from the fronds of the above seaweeds according to the method of Drummond et al.[9] as described in a previous paper[3]. Electrophoresis in 0.1 M HCl[10] and 0.1 M zinc acetate[1] showed each alginate as a single band. The preparations were stored at ca. −20 to −25°C until used.

Estimation of molecular weight.—The molecular weight of each alginate was estimated from its degree of polymerization, which was calculated from the intrinsic viscosity by using the formula of Donnan and Rose[11], $dp/[\eta] = 58$. Viscosity measurements were performed on 0.1 M NaCl solution (0.1–0.5 mg mL^{-1}) of the polyuronides with an Ostwald type viscosimeter at 25.00 ± 0.02°C.

^{13}C NMR spectrometric estimation of the monomeric and block compositions of M and G in alginates.—Monomeric and block compositions of M and G were estimated from the ^{13}C NMR spectra of slightly depolymerized alginates by the method of Grasdalen et al.[12] as previously described[3]. The slightly depolymerized alginates were prepared by mild hydrolysis with HCl (pH 3.0) for 30 min at 100°C.

Determination of Ca^{2+} in alginates.—One mL of 60% HNO_3 was added to ca. 2–3 mg of the alginate that was dried in vacuo, and then the suspension was hydrolyzed for 7 h at 120°C. The content of calcium in the hydrolysate was estimated using an atomic absorption spectrophotometer (Hitachi; type 170-10), with $CaCl_2$ (analytical grade, Nakarai Chemical, Co.) as the standard.

Preparation of macrophages.—A dose of 50 mg kg^{-1} of an alginate as the sample was injected i.p. to a normal male mouse (ICR, 6 weeks old, body weight, 32 g) once a day for 4 days. Then, on the day after the last injection of the sample, 1.5 mL of 10% proteose peptone (DIFCO Laboratories, Detroit MI) was further injected i.p. to the mouse to induce peritonial macrophages. Three days thereafter, a peritoneal exudate cell suspension was collected from the mouse after injection of 5 mL of RPMI1640 medium (containing 10% FCS). After centrifugation of the suspension, the precipitated cells were washed twice with Hank's medium and then resuspended in RPMI1640 medium (10% FCS).

Chemotaxis assay.—The assay was carried out according to the modified method[13] of Horwitz and Garrett[14] with a Blind well chamber 200 μL of 1 mM chemotactic peptide[15] [N-formyl-methionylleucylphenylalanine (Sigma)] solution in RPMI1640 (10% FCS) was placed in the bottom of the chamber, and then the bottom well was covered with a polyvinylpyrrolidone (PVP) free polycarbonate

filter[16] with 5 μm holes (Nuclepore, Pleasanton, CA). Then the top well of the chamber was screwed onto the bottom one and filled with 0.8 mL of macrophage (8×10^5 cells mL^{-1}) suspension in the medium. The chamber was incubated for 4 h at 37°C in humidified air containing 5% CO_2, and then the filter was removed and the nonmigrated cells on the filter were scraped off from its top side. The filter was mounted on a glass slide with egg yolk–glycerol solution and stained with diluted Giemza staining solution (E. Merck), and then the migrated cells were counted in five fields under a microscope. The number of the cells was averaged. A physiological saline solution alone was used instead of alginate solution as the control. The chemotactic response of the macrophages from the treated mouse was expressed as the ratio to the chemotaxis (100) of the control.

CD spectrum.—Circular dichroism (CD) measurements were made in a 1-cm cell on an aq alginate solution (1.0 mg mL^{-1}) in the presence and the absence of Ca^{2+} using JASCO J-500C spectropolarimeter with a DP-501N Data Processor. The sample solutions were kept at pH 7.

Assay of antitumor activity.—Antitumor activity of the alginates was assayed against Sarcoma-180 tumor (ascitic type) cells (1×10^5) implanted in ICR mice (5 weeks old) as previously[3] described. The ascites Sarcoma-180 tumor cells used were kindly donated by The Kitasato Institute, Tokyo. The test samples, dissolved in physiological saline solutions with or without calcium chloride (1–3 mM), were injected i.p. in doses of 25 mg kg^{-1} (injection volume, 0.25 mL) and 50 mg kg^{-1} (injection volume, 0.5 mL) at 24-h intervals on the days described in Table III after implantation of the tumor. Antitumor activity was evaluated by the increase in life span (ILS). Survival of mice was scored 60 days after implantation of tumors, and mice remaining alive after this observation period were considered to be cured.

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