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Suppression of eosinophilia by *Petalonia binghamiae* polysaccharides may relate to their eotaxin-binding ability

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Abstract

We have reported that *Petalonia binghamiae* polysaccharides suppressed the delayed-type hypersensitivity (DTH) via a Toll-like receptor (TLR) 4 signal. Treatment with *Petalonia binghamiae* polysaccharides significantly suppressed DTH and eosinophilia in C3H/HeN mice but not TLR-4 mutant C3H/HeJ. *Petalonia binghamiae* polysaccharides but not alginic acid or fucoidan suppressed the DTH in C3H/HeN mice in either oral or intraperitoneal administration. The number of eosinophils at the site of inflammation was decreased significantly by the administration of *Petalonia binghamiae* polysaccharides but not by that of alginic acid or fucoidan. In this study, we examined whether *Petalonia binghamiae* polysaccharides can bind to eotaxin, CCL17, and CCL27. CCL17 is a thymus and activation-regulated chemokine for T lymphocytes involved in allergic reactions in the skin and CCL27 is a skin-associated chemokine. We found that fucoidan showed a higher affinity to eotaxin and CCL27 compared with *Petalonia binghamiae* polysaccharides and heparin. *Petalonia binghamiae* polysaccharides showed a higher affinity to CCL17 than fucoidan or heparin. In conclusion, *Petalonia binghamiae* polysaccharides, fucoidan, and heparin may suppress the migration of eosinophils towards eotaxin *in vitro*, in part, by binding to eotaxin. Since the binding ability of algae polysaccharides to eotaxin does not always correlate with the degree of suppression of DTH, this is one of the criteria that we can use for selecting materials before applying them *in vivo*.

Key words: *Petalonia binghamiae*, polysacchardides, eosinophils, eotaxin, TLR4, surface plasmon resonance analysis

Introduction

We have analyzed the suppressive effects of algae polysaccharides on the delayed-type hypersensitivity (DTH) against 2, 4, 6-trinitrochlorobenzene (picryl chloride, PCl) (Tominaga *et al*., 2010, 2011). In particular, we focused on the effects of algae polysaccharides (PS) on eosinophil migration to the site of inflammation where eosinophil chemoattractant, eotaxin, is produced. One of the interesting things is that *Petalonia binghamiae* (*P. binghamiae*) PS and heparin suppressed the DTH response in C3H/HeN mice but not in C3H/HeJ mice, TLR-4 mutants. Indeed, *P. binghamiae* PS induce NFκB in response to TLR 4 (Tominaga *et al*., 2010). Although alginic acid from *Macrocystis pyifera* (Kelp) or fucoidan from *Fucus vesiculosus* also induce NFκB in response to TLR 4, they do not suppress the DTH response in either C3H/HeN mice or C3H/HeJ mice, TLR-4 mutants. Among these algae polysaccharides, only *P. binghamiae* PS can induce NFκB in response to TLR 2. At this moment, we do not know the reason why *P. binghamiae* PS suppressed the DTH response.

Up until this point in time we used a DTH model that induces eosinophilia and a chemokine eotaxin that attracts eosinophils to the site of allergy-inflammation (Satoh *et al*., 1997). Indeed, the level of eotaxin in the ears, where the antigen PCl is painted two weeks after sensitization, increased

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and decreased through painting hydrocortisone or an anti-allergic reagent such as peridinin (Onodera *et al*., 2014).

Since DTH responses are caused mainly by T lymphocytes, macrophages, and eosinophils, we also focused on other skin-associated chemokines such as CCL-17 and CCL27. CCL17/TARC (thymus and activation-regulated chemokine) is constitutively expressed in the thymus and produced by dendritic cells, endothelial cells, keratinocytes, and fibroblasts and is classified as a Th2 type chemokine that dominates in allergic reaction such as atopic dermatitis or asthma (Saeki *et al*., 2006). In atopic dermatitis model NC/Nga mice, CCL17 is highly expressed in the basal epidermis of lesional skin, but not in non-lesional skin, and the high level of CCL17 expression disappeared synchronously with the healing of lesional skin, when these mice were treated with steroid ointment (Saeki *et al*., 2006). Indeed, they reported that the serum levels of CCL17 in atopic dermatitis patients were significantly higher than those of healthy control or Th1-dominant patients with psoriasis vulgaris, inflammatory skin diseases in which IFN- γ producing cells dominate. They also noted that CCL17 receptor CCR4 expression in $CD4^+$ T lymphocytes in peripheral blood and in $CD4^+$ mononuclear cells in the epidermis increased in atopic dermatitis patients more than expression in healthy and psoriasis subjects. These results suggest that the CCL17-CCR4 system plays an important role in skin allergic diseases such as atopic dermatitis.

It is reported that the skin-associated chemokine CCL27 and its receptor CCR10 mediate chemotactic responses of skin-homing T cells *in vitro* (Homey *et al*., 2002). Homey *et al*. also reported that most skin-infiltrating lymphocytes in patients suffering from psoriasis, atopic or allergic-contact dermatitis express CCR10 and that epidermal basal keratinocytes produced CCL27 protein that bound extracellular matrix, mediated adhesion and was displayed on the surface of dermal endothelial cells. They also reported that intracutaneous CCL27 injection attracted lymphocytes and neutralization of CCL27-CCR10 interactions impaired lymphocyte recruitment to the skin leading to the suppression of allergen-induced skin inflammation.

In this report, we examined the direct binding of algae polysaccharides to eotaxin, CCL17, and CCL27 to make a rapid test to determine whether candidate polysaccharides are useful for the inhibition of DTH.

Materials and methods

1) Delayed-type hypersensitivity assay

C3H/HeN mice were purchased from CLEA Japan INC. (Osaka, Japan). Female 8 weeks old mice were used in all experiments. All experiments were performed in a specific pathogen free condition under the ethical guidelines of Kochi University. Two days before sensitization with 2, 4, 6-trinitrochlorobenzene (picryl chloride, PCl from Nakarai Tesque Inc., Kyoto, Japan), cyclophosphamide (purchased from Shionogi Company, Osaka, Japan) was injected subcutaneously (150 mg/kg in DW) to remove proliferating immunosuppressive cells (Satoh *et al.*, 1997). After removing coat hair, the mice were immunized by painting their abdominal skin with 0.05 ml of 7% PCl in ethanol: acetone (3: 1). Two weeks after sensitization, 0. 02 ml of 1% PCl in acetone: olive oil (1: 4) was painted on each ear lobe to challenge the mice. Ear thickness was measured with a dial thickness gauge (Peakock, Tokyo, Japan) before and after challenge and was expressed as the mean increment in thickness (swelling) for each ear.

2) Polysaccharides

The Molecular Mass (MS) of each polysaccharide are as follows. Alginic acid prepared from *Macrocystis pyrifera* (kelp): mixed polymer of mannuronic acid and guluronic acid (Sigma-Aldrich Inc., St. Louis, MO), MS: 420,100 − 420,700. Fucoidan was purchased from *Fucus vesiculosus* (Sigma-Aldrich), MS: 841,388 ? 1,113, 156. *Petalonia binghamiae* polysaccharides were prepared as 66% ethanol precipitates from hot water extracts, MS: 273,555 − 353,476. Heparin sodium salt was purchased from MP Biomedicals, LLC (Solon, OH), MW: ~ 3000.

3) Chemokines

Eotaxin was purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). CCL17 and CCL27 were purchased from R&D Systems, Inc. (Minneapolis, MN)

4) Polysaccharides binding to chemokines

BIA core J and Chip CM5 (Pharmacia Biosensor AB, Uppsala, Sweden) was used for the binding assay of polysaccharides to eotaxin, CCL17, and CCL27. All chemokines were bound to Sensor Chip CM5 according to the method described (Karlsson *et al.*, 1997). In brief, the carboxyl groups on the surface of sensor chips were activated by a solution containing 0.2 M *N*-ethyl-*N*'-(3-dimethylamino-propyl) carbodiimide (EDC) and 0. 05 M *N*-hydroxysuccinimide. Eotaxin, CCL17, and CCL27 were bound to the surface of sensor chips by injecting 20 μl of 100 μg/ml of each chemokine in 10 mM acetate buffer at pH 4.7-5.1. To block the remaining ester groups, 1 M ethanolamine hydrochloride was injected for 7 min.

5) Kinetic analysis of polysaccharides interaction with eotaxin, CCL17, and CCL27

Hepes-buffered saline (10 mM Hepes with 0.15 M NaCl, 3.4 mM EDTA, and 0.005% surfactant P20 at pH7.4) was used as a running buffer. The binding of polysaccharides (alginic acid, fucoidan, *P. binghamiae* PS, heparin) to each chemokine was analyzed by injecting each polysaccharide at the flow rate of 30 μl/min at the indicated concentration in figures (Fig. 3, Fig. 4, Fig. 5). After each application of polysaccharides, sensor chips were washed with 0.01 − 0.05% SDS to remove residual binding.

For analyzing the data, BIA evaluation software was used according to the procedure recommended by the manufacturer.

Results and Discussion

To observe the effects of algae polysaccharides the thickness induced by the DTH response to PCl was examined. Typical pathology of ear thickness is shown in Figure. 1. In normal mice, ear thickness is about 250 μm. However, in the DTH response against PCl, the thickness is increased up to about 400 μm in response to the antigen challenge two weeks after the sensitization (immunized mice by painting PCl on the abdominal skin). In this Figure, negative (-) control is the section of an ear from a non-challenged mouse after sensitization and the positive (+) control is the section of an ear of a mouse that is prepared 48 hours after being challenged two weeks after sensitization. *P. binghamiae* PS suppressed the ear thickness when administered either orally or intraperitoneally (i. p.). In contrast, alginic acid did not suppress the ear thickness significantly regardless of the way of administration.

Figure 2 shows the ear sections of a negative control and a positive control. In the positive control, eosinophils whose cytoplasm is stained red with eosin are indicated by yellow arrows; these increased significantly 48 hours after the antigen challenge. There were no such cells in the sections from a negative control mouse or a normal mouse.

One of the major characteristics in symptoms of atopic dermatitis is the increase of eosinophils in the peripheral blood and the infiltration of eosinophils to the site of inflammation. To examine the effects of polysaccharides on the migration of eosinophils toward eotaxin, we applied algae polysaccharides to the *in vitro* migration inhibition assay toward eotaxin as described (Onodera *et al*., 2014). We observed that alginic acid, carrageenan, fucoidan, and heparin suppressed the migration of eosinophils toward eotaxin *in vitro*, although the inhibitory effects of carrageenan was less than those of the other polysaccharides (Table 1, Table 2). We thought that the direct binding of polysaccharides to eotaxin may be a good criterion to estimate the inhibitory effects of polysaccharides on DTH response *in vivo.*

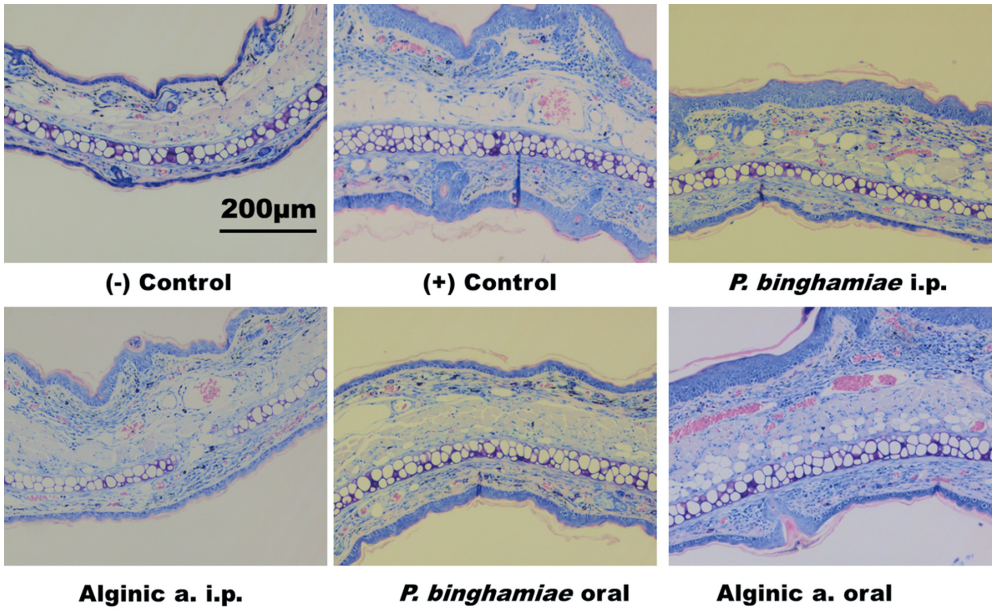

Fig.1. Effects of polysaccharides on ear thickness in C3H/HeN at 48 h after challenge.
DTH response was elicited against PCl as described in Materials and methods. Polysaccharides used in this assay were alginic acid and *P. binghamiae* PS. Each reagent was injected intraperitoneally into a mouse at a dose of 200 μg/0.1 ml saline/mouse three hours before antigen challenge. Alternatively, polysaccharides were administered orally 1 mg/mouse every other day for two weeks after sensitization with antigen. Forty eight hours after the antigen challenge, ears were fixed with 4% paraformaldehyde in a 0.1 M Phosphate buffer, pH7.2 and tissue sections were stained with hematoxylin and eosin.

Table 1. Comparison of regulatory effects of algae polysaccharides on the migration of eosinophils between *in vitro* and *in vivo* and their activities to TLR2 and TLR4.

Polysaccharides	NFκB induction in response to	*In vitro* migration to eotaxin	*In vivo* DTH		
			C3H/HeN		C3H/HeJ TLR4 mutant
			Orally	i.p.	i.p.
Alginic acid	TLR4: ⇈ TLR2: →	⇊	→	→	→
Carrageenan (ι, λ)	TLR4: ↑ TLR2: →	↓	ND	↓ (BALB/c)	ND
Fucoidan	TLR4: ⇈ TLR2: →	⇊	→	→	→
Petalonia binghamiae PS	TLR4: ⇈ TLR2: ↑	⇊	↓	↓	→
Heparin	ND	⇊	ND	↓	→
Hyaluronic acid	ND	ND	→	→	ND
Spirulina pacifica complex polysaccharides	TLR4: ↑ TLR2: ↑	⇊	ND	↘	→

i.p.: intraperitoneally, ND: not done.

Table 2. Comparison of affinity of algae polysaccharides to eotaxin, CCL17, and CCLL27.

Exp. 1.

Polysaccharides	Effects on Eo migration to eotaxin (*in vitro*)	Effects on DTH (i.p.) (*in vivo*)	Affinity to chemokines (Kd)		
			Eotaxin	CCL17	CCL27
Fucoidan	⇊	→	14.9 pM	51.3 pM	12.7 pM
Petalonia binghamiae	⇊	↓	4.02 nM	10.4 pM	4.27 nM
Heparin	⇊	↓	3.73 nM	0.94 nM	0.557 nM

Kd: Dissociation constant. Eo: eosinophils.

Exp. 2.

Polysaccharides	Effects on Eo migration to eotaxin (*in vitro*)	Effects on DTH (i.p.) (*in vivo*)	Affinity to chemokines (Kd)		
			Eotaxin	CCL17	CCL27
Alginic acid	⇊	→	ND	ND	ND
λ–Carrageenan	↓	↓	4.2 nM	4.78 pM	4.91 pM
κ–Carrageenan	↓	ND	ND	ND	ND
ι–Carrageenan	↓	↓	4.16 nM	32.1 pM	5.41 pM

Kd: Dissociation constant. ND: not done. Eo: eosinophils.

Supplementary data. Interaction of polysaccharides with chemokines.

Polysaccharides	Resonance Unit (RU) at the concentration of 100 μg/mL		
	Eotaxin	CCL17	CCL27
Fucoidan	17.3	262.5	186.2
Petalonia binghamiae	76	180.7	198.8
Heparin	37.8	108.5	93.2
Alginic acid	2.7	23.2	43.2
λ–Carrageenan	23.4	126.6	152.7
ι-Carrageenan	13.7	181.7	149.9
Hyaluronic acid	1.5	10.2	1.2

Data used for the calculation for Kd.
The data shown in Fig. 3, 4, 5 are one of the typical data among several independent experiments.

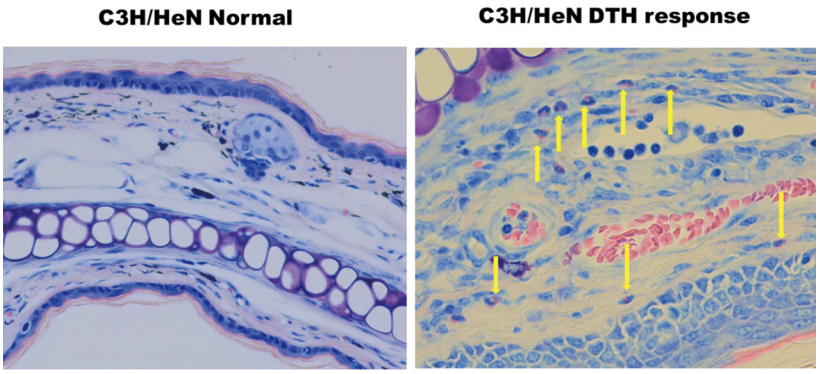

Fig.2. Migration of eosinophils to the site of inflammation in the DTH response to picryl chloride.
Typical infiltration of eosinophils were shown in the ear section of C3H/HeN mouse that was sensitized and challenged with PCl. Ear sections were fixed and stained as shown in Fig.1 except that they are two-fold magnified.

Another reason we thought of this assay is that recently we are having an increasing demand from an animal rights society to reduce our animal experiments. We thought that this could be a first screening before administrating polysaccharides to the model mice for evaluating their effects on DTH responses.

Reactions between polysaccharides and chemokines were analyzed by optical biosensors, BIAcore J. At first, we examined the binding affinity of each polysaccharides to eotaxin (Figure 3). We found that fucoidan had the highest affinity for eotaxin, and *P. binghamiae* PS, heparin, and carrageenan (ι and λ) had similar affinity for eotaxin at a lower level (Table 2). The binding ability of fucoidan, *P. binghamiae* PS, heparin, and carrageenan (ι and λ) to eotaxin may explain at least, in part, their inhibitory effects on the migration of eosinophils toward eotaxin *in vitro*. Although alginic acid did not bind to eotaxin, it suppressed the migration of eosinophils toward eotaxin, suggesting that alginic acid directly acts on eosinophils to stop the migration. Because this migration assay is an hour assay, the inhibitory effects on gene expression cannot be involved. Inhibitory effects may be those such as the inhibitory effects on Ca^{++} release from endoplasmic reticulum or mitochondria, or on actin polymerization. Indeed, we have observed the inhibitory effects of *P. binghamiae* PS on the increase of Ca^{++} concentration in the cytoplasm (data not shown).

P. binghamiae PS and λ -carrageenan showed a higher affinity to CCL17 than those of fucoidan, ι-carrageenan, and heparin (Figure 4, Table 2). Although *P. binghamiae* PS, λ-carrageenan, ι-carrageenan, and heparin suppressed the DTH response significantly, fucoidan did not suppress the DTH response (Table 1, Table 2). Even though we cannot simply

rely on the binding assay of polysaccharides to chemokines, the low affinity of fucoidan for CCL17 among high molecular weight polysaccharides excepting heparin may explain at least, in part, the lowest inhibitory effect of fucoidan on the DTH response, because the important role of CCL17 in atopic dermatitis is well established (Saeki *et al.*, 2006).

Fucoidan had a higher binding affinity to CCL27 than P. *binghamiae* PS. *P. binghamiae* PS had about a 300-fold less affinity compared to that of fucoidan (Figure 5, Table 2). Both ι-carrageenan and λ-carrageenan had an even higher affinity to CCL27 compared with fucoidan. Because anti-CCL27 antibodies suppressed the skin-infiltrating lymphocytes and impaired allergen-specific skin inflammation compared with isotype-matched control antibodies (Homey *et al.*, 2002), these polysaccharides can suppress the DTH, if they bind to CCL27 like antibodies. This is not the case for fucoidan,

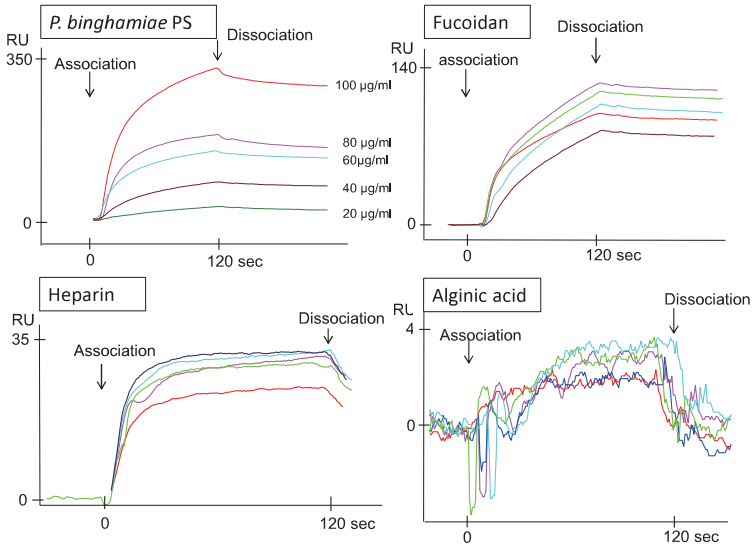

Fig.3. Kinetic analysis of the interaction between algae polysaccharides and eotaxin.
Biacore analyses were performed for kinetic analysis of eotaxin and polysaccharides, *P. binghamiae* PS, fucoidan, heparin, and alginic acid as described in Materials and methods. Binding of these polysaccharides to eotaxin is expressed as a resonance unit (RU) in a linear scale as a vertical axis during the time period indicated as a horizontal axis.

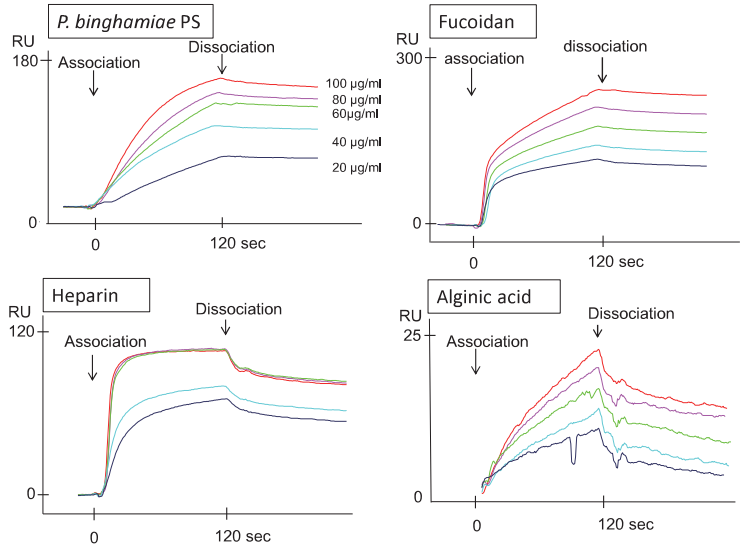

Fig.4. Kinetic analysis of the interaction between algae polysaccharides and CCL17.
Biacore analyses were performed for kinetic analysis of CCL17 and polysaccharides, *P. binghamiae* PS, fucoidan, heparin, and alginic acid as described in materials and methods. Binding of these polysaccharides to CCL17 is expressed as a resonance unit (RU) in a linear scale as a vertical axis during the time period indicated as a horizontal axis.

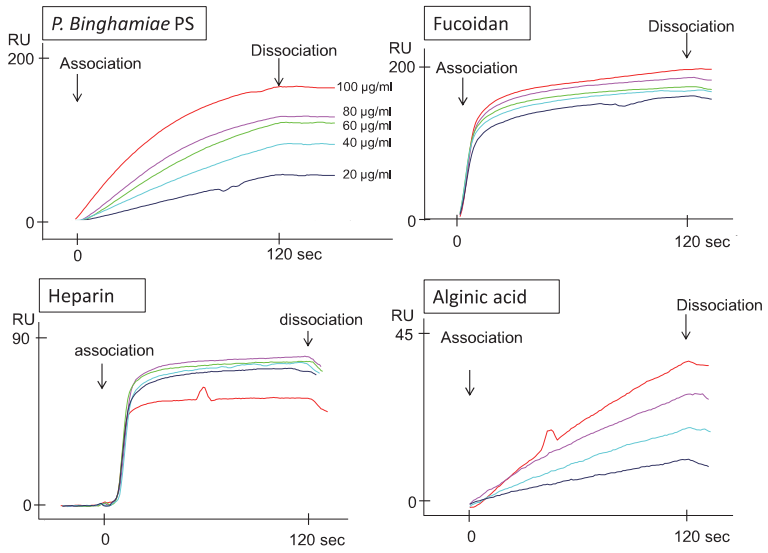

Fig.5. Kinetic analysis of the interaction between algae polysaccharides and CCL27.
Biacore analyses were performed for kinetic analysis of CCL27 and polysaccharides, *P. binghamiae* PS, fucoidan, heparin, and alginic acid as described in materials and methods. Binding of these polysaccharides to CCL27 is expressed as a resonance unit (RU) in a linear scale as a vertical axis during the time period indicated as a horizontal axis.

because it does not suppress the DTH response. Fucoidan may be trapped by other cells or molecules before encountering CCL27 *in vivo*.

From the results described above, we cannot explain why heparin, which has a lower affinity to eotaxin, CCL17, and CCL27 than fucoidan, can suppress the DTH efficiently. The degree of suppression by polysaccharides seems to be in accordance with the resonance units of each polysaccharides to eotaxin at the concentration of 100 μg/mL (Table 2, supplementary data). Both heparin and *Petalonia binghaminae* polysaccharides have higher level of binding to eotaxin compared with other polysaccharides.

Lortat-Jacob et al. reported that many of the chemokines can bind to heparan sulfate. They proposed that four docking modes are involved in the interaction between chemokine dimers and heparin or heparan sulfate: one mode for each of the CC and CX_3C types of chemokines and two different modes within the CXC family. In each case, different clusters of basic amino acids can be defined as an arrangement of amino acids at the protein surface that provides efficient binding of heparan sulfate (Lortat-Jacob *et al*., 2002).

Parish summarized by noting that heparan sulfate is involved in the initial adhesion of leukocytes to the inflamed endothelium, the subsequent chemokine-mediated transmigration through the vessel wall and the establishment of both acute and chronic inflammatory reactions (Parish, 2006). Nelson *et al.* reported that heparin oligosaccharides bind L- and P-selectin and inhibit acute inflammation by diminishing the influx of neutrophils into the peritoneal cavities of thioglycollate-treated mice when administered intravenously (Nelson *et al*., 1993). Wang *et al*. concluded that (a) heparin's anti-inflammatory effects are mainly mediated by blocking P- and L-selectin-initiated cell adhesion; (b) the sulfate groups at C6 on the glucosamine residues play a critical role in selectin inhibition; and (c) some non-anticoagulant forms of heparin retain anti-inflammatory activity. Such analogs may prove useful as therapeutically effective inhibitors of inflammation (Wang *et al.*, 2002).

Syndecan is a type I integral membrane heparan sulfate proteoglycan involved in inflammation. It is expressed on resting lymphocytes, monocytes, neutrophils, eosinophils, macrophages, proliferating and/or activated T cells, pre-B cells, plasma cells, and platelets (Parish, 2006). Syndecan-4 binds to basic fibroblast growth factor, midkine, and tissue factor pathway inhibitor via its heparin sulfate chains. In the kidney, syndecan-4 is expressed in the ureteric bud invaginating into the metanephric mesenchyme at 11. 5 gestational days, and remains in the collecting ducts, distal renal tubules, glomeruli, and some capillaries between renal tubules (Ishiguro *et al.*, 2001). Ishiguro *et al*. reported that although most renal functions of syndecan-4 deficient ($Synd4^{-/-}$) mice were not impaired, a significant increase in susceptibility to κ-carrageenan-induced renal damage was observed in these mice (Ishiguro et al., 2001). κ-Carrageenan was heavily deposited in the collecting ducts of syndecan-4 deficient mice and caused obstructive nephropathy, leading to death of 30% of the mice within a week after administration of κ-carrageenan. They interpreted the results in the following way: syndecan-4 expressed in the collecting ducts may prevent the deposition of κ-carrageenan (Ishiguro *et al.*, 2001).

Kanabar *et al*., reported that heparin and structurally related polymers attenuate eotaxin-1 (CCL11) release from human airway smooth muscle. They also found that N-desulfated, 20% re-N-acetylated heparin was devoid of activity against IL-13-dependent eotaxin-1 production and 90% re-N-acetylation in the molecule restored attenuating activity similar to unfractionated heparin against IL-13-stimulated eotaxin-1 release (Kanabar *et al*., 2008). These results suggest that N-sulfation is required for heparin' s eotaxin-1 attenuating activity.

Another interesting thing is that *Spirulina* complex polysaccharides can suppress the DTH response *in vivo*. Both *P. binghamiae* polysaccharides and *Spirulina* complex polysaccharides share the ability to produce NFκB in response to TLR2 and TLR4 (Table 1).

Conclusion

P. binghamiae PS, fucoidan, carrageenan (λ and ι) and heparin bound to eotaxin and suppressed the migration of eosinophils toward eotaxin *in vitro*. Although alginic acid did not bind to eotaxin significantly, it suppressed the migration of eosinophils toward eotaxin, suggesting that alginic acid suppressed the migration of eosinophils toward eotaxin by acting on eosinophils.

Although fucoidan has a much higher affinity for eotaxin compared with *P. binghamiae* PS and heparin, it did not suppress either the eosinophilia or the DTH response, suggesting the importance of the processing of these polysaccharides *in vivo.*

We can use the ability of polysaccharides to bind to eotaxin, or other chemokines as one of the criteria before applying them *in vivo* experiment. However, since the effectiveness of each polysaccharide to suppress the DTH response depends on many factors *in vivo,* we cannot simply rely on the ability of each polysaccharide to bind to eotaxin to estimate the effectiveness of polysaccharides to suppress the eosinophila or DTH. We think that this direct binding assay of polysaccharides to each chemokine can supply us with valuable information.

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