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Effects of Edible Algae on Immune Responses: Algae Polysaccharides Regulate Delayed-type Hypersensitivity and Tumor Growth

Akira Tominaga[1*], Teruyuki Fujii[1], Hiromi Okuyama[2], Takahiro Taguchi[1], Yutaka Kusumoto[2] and Shiro Ono[2]

[1] Laboratory of Human Health and Medical Science, Graduate School of Kuroshio Science, and Department of Molecular and Cellular Biology, Kochi Medical School, Kochi University, Kohasu, Okoh-cho, Nankoku, Kochi 783-8505 Japan,

[2] Laboratory of Immunology, Faculty of Pharmacy, Osaka Ohtani University, 3-11-1, Nishikiorikita, Tondabayashi, Osaka 584-8540, Japan

Abstract

Algae are eaten as health food in many Asian countries. We summarize our recent results regarding the immunoregulatory role of polysaccharides from edible algae on delayed-type hypersensitivity (DTH) and antitumor immune responses using mice. These algae are divided into two types; those which manipulate immune responses through toll-like receptor 4 (TLR 4), and those which use different receptors to modify the immune response. *Petalonia binghamiae* polysaccharides attenuate DTH and tumor growth by reducing the production of inflammatory cytokine, IL-17 through TLR4. The serum level of IL-17 was decreased significantly, only when *P. binghamiae* polysaccharides were administered intraperitoneally in the DTH response. Intraperitoneal administration of other brown algae polysaccharides, such as alginic acid did not suppress the DTH response nor reduced the serum level of IL-17. Although orally administered *P. binghamiae* polysaccharides did not reduce the serum level of IL-17, they inhibited the migration of eosinophils. These results suggest that the inhibition of either the generation of IL-17 or the migration of eosinophils is effective in regulating inflammation, and that these two events were separately regulated. Here, we present data suggesting that either the peritoneal or oral administration of *P. binghamiae* polysaccharides efficiently inhibits the migration of eosinophils to the site of inflammation in a DTH model using IL-5 transgenic mice. Alginic acid suppressed tumor growth in both C3H/HeN and C3H/HeJ mice and did not suppress the DTH response as strongly as *P. binghamiae* polysaccharides. We propose the potential usefulness of edible algae as fine tuning reagents of the immune responses.

Key words: algae, delayed-type hypersensitivity, auto-tumor immunity

1. Introduction

We have explored the immunoregulatory roles of polysaccharide fractions derived from edible sea algae. To explore the responsiveness to TLR2 and TLR4, we used Golenbock's cells, 7.19/TLR2, and 3E10, respectively (Delude *et al.,* 1998, Medvedev *et al.,* 2001). Among the algae polysaccharides screened, we chose the polysaccharides from brown alga, *Petalonia binghamiae* (*P. binghamiae*) and compared their immunoregulatory roles with commercially available polysaccharides such as fucoidan and alginic acid. *P. binghamiae* polysaccharides (66% ethanol precipitates from hot water extracts) were unique in terms of their responsiveness to TLR2 and TLR4. Compared with alginic acid and fucoidan, *P. binghamiae* polysaccharides induced NF-κB in response to either TLR2 or TLR4 weakly or to the equivalent level (Tominaga *et al.,* 2010). Fucoidan and alginic acid are reported to generate NF-κB strongly in response to TLR4 but not at all or only marginally in response to TLR2.

We examined the effects of these polysaccharides on regulation of delayed-type hypersensitivity (DTH) and for their anti-tumor effects using mice. *P. binghamiae* polysaccharides suppressed DTH responses against picryl chloride when administered either orally or intraperitoneally (i.p.), and also suppressed the growth of hepatocellular carcinoma. Both suppressions were observed in C3H/HeN mice but not in C3H/HeJ TLR4 mutant

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*Corresponding author: e-mail tominaga@kochi-u.ac.jp

mice.

Major factors that induce DTH reactions are T lymphocytes and macrophages as well as inflammatory cytokines such as IFN-γ and IL-17. We will discuss not only these factors, but also mention the role of eosinophils in the DTH response using transgenic mice which produce IL-5 continuously given control of the metallothionein promoter, resulting in the proliferation of eosinophils. The percentage of eosinophils among leukocytes in peripheral blood is about 50% (Tominaga *et al.,* 1991). In normal mice, the percentage of this leukocyte is less than 3%. In this study, we found that both IL-17 and eosinophils are important factors in DTH reactions to enhance immune responses. IL-17 and IFN-γ are also important factors in regulating tumor growth, although their engagement in the antitumor activity depends on the type of tumor cells. Production of these cytokines and the migration of eosinophils to the site of inflammation were manipulated by the edible algae polysaccharides.

2. Materials and Methods

1) Animals

C3H/HeJ (TLR4 mutant) mice were purchased from CLEA Japan INC. (Osaka, Japan). Female mice, 8-10 weeks old, were used in all experiments. IL-5 transgenic mice (C3H/HeN-TgN(IL-5)-Imeg) were developed by ourselves (Tominaga *et al.,* 1991) and maintained in our animal facility in a specific pathogen free condition. Female IL-5 transgenic mice, 8-15 weeks old, were also used. All experiments were performed under the ethical guidelines of Kochi University.

2) Chemicals

Picryl chloride (PCl; 2,4,6-trinitrochlorobenzene) was purchased from Nacalai Tesque Inc. Cyclophosphamide (CY) was purchased from the Shionogi Company, Osaka, Japan.

3) Algae polysaccharides

Molecular Mass (MS) of each polysaccharides: Fucoidan was prepared from *Fucus vesiculosus* (Sigma-Aldrich), MS: 841388 ~ 1113156. Alginic acid was prepared from *Macrocystis pyrifera* (kelp); mixed polymer of mannuronic and guluronic acid (Sigma-Aldrich), MS: 420100 ~ 420700. *Petalonia binghamiae* (*P. binghamiae*) polysaccharide (66% ethanol precipitates from hot water extracts), MS: 273555 ~ 353476. *Spirulina pacifica* (*S. pacifica*) complex polysaccharides (lipopolysaccharide Westphal fraction was prepared by Dr. Satoshi Fukuoka, AIST, Takamatsu, Japan), MS: 1284 ~ 28315.

4) Sensitization and challenge

Two days before immunization with PCl, cyclophosphamide 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 immunization, 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 (Peacock, Tokyo, Japan) before and after challenge and was expressed as the mean increment in thickness (swelling) of each ear in micrometers.

5) Administration of algae polysaccharides

Three hours before the antigen challenge, each polysaccharide fraction (150, 200, 300, 500 μg/0.1 ml saline/mouse) was administered to the mice as indicated in each figure legend in case of intraperitoneal injection. Oral administration was done every other day after immunization with a capillary (1 or 2 mg/0.2 ml distilled water/mouse) for two weeks until the antigen challenge.

3. Results

1) Algae polysaccharides suppress the DTH response

We have found that *P. binghamiae polysaccharides* attenuate the DTH in a toll-like receptor (TLR) 4 dependent manner (Fig. 1). *P. binghamiae* polysaccharides suppressed the DTH response in mice when administrated either orally or intraperitoneally (i.p.) in C3H/HeN but not in the C3H/HeJ TLR4 mutant, which is unable to transmit signals through TLR4, because this strain has a mutation at the cytoplasmic domain of TLR4 (Poltorak *et al.,* 1998). In contrast, alginic acid did not suppress the DTH response in either strain of mice. Interestingly, heparin, a mucopolysaccharide sulfuric acid ester, that is made by mast cells and basophils and has the highest amount of iduronic acid and of N- and O-sulfate residues, did not respond to either TLR2 or TLR4 in terms of NF-κB generation assay using Golenbock's cells. It suppressed the DTH response only in C3H/HeN but not in C3H/HeJ (Fig. 1). This suggests that heparin may use the common signaling pathway with *P. binghamiae* polysaccharides in relation to TLR4.

There are two potent cytokines, IL-17 and IFN-γ that are engaged in inflammation. We have already reported that both IFN-γ and IL-17 are involved in the DTH response against picryl chloride (PCl; 2,4,6-trinitrochlorobenzene)(Tominaga *et al.,* 2010). We have also reported that the level of IL-17 was down-regulated

to one third of the control group (i.p. administration of alginic acid) when *P. binghamiae* polysaccharides were administered i.p. but not orally (Tominaga *et al.,* 2010). Therefore, we sought to discover how the oral administration of *P. binghamiae* polysaccharides suppressed the DTH response against picryl chloride.

There are lymphocytes and macrophages which migrate to the site of inflammation in the DTH response. Besides these cells, we found that eosinophils were increased at the site of inflammation. Migration of eosinophils to the site of inflammation is one of the features of DTH response. Here, we have focused on eosinophils using IL-5 transgenic mice which contain about 50% eosinophils among white blood cells in peripheral blood (Tominaga *et al.,* 1991). This strain does not have eosinophils at the site of inflammation without immunization and challenge with PCl.

2) Effects of algae polysaccharides on DTH in IL-5 transgenic mice, C3H/HeN-TgN (IL-5)-Imeg

We examined how long the DTH response lasted in three strains of mice. In both C3H/HeJ and C3H/HeN mice, the DTH response against PCl lasted about 10 days (Fig. 1). In IL-5 transgenic mice which have the same genetic background as C3H/HeN, however, it lasted 18 days and the ear swelling in the positive control was about 150 μm (Fig. 2). In contrast, ear swelling was about 100 μm in both C3H/HeJ and C3H/HeN (Fig. 1). *P. binghamiae* polysaccharides suppressed the DTH response in IL-5 transgenic mice, either 24 or 48 hours after the antigen challenge in either route of administration, i.p. or oral. Although alginic acid and fucoidan weakly suppressed the DTH response 24 hours after the antigen challenge, they did not suppress the DTH response after 48 hours (Fig. 2).

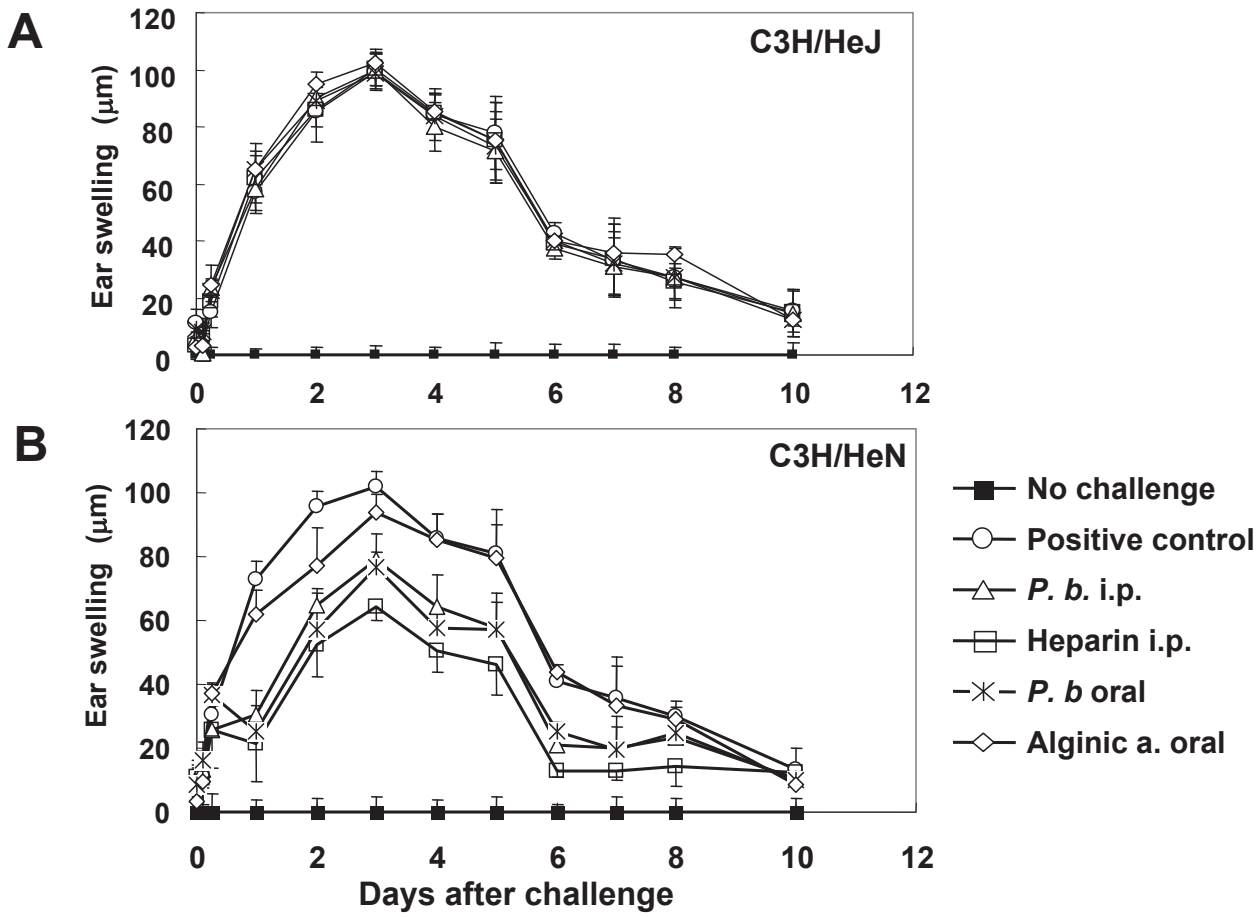

Figure 1. Suppression of DTH responses by polysaccharides in C3H/HeJ and C3H/HeN.
DTH response was elicited against PCl as described in the section on materials and methods. Reagents used to control the DTH response were *P. binghamiae* polysaccharides, heparin, and alginic acid. Each reagent was injected intraperitoneally (i.p.) into a mouse at a dose of 200 μg/0.1 ml saline/mouse 3 hours before antigen challenge. In other cases, reagents were administered orally 1 mg/mouse every other day for two weeks after immunization with antigen. *P. b.: Petalonia binghamiae*. **Panel A**: DTH response against PCl in C3H/HeJ. **Panel B**: DTH response against PCl in C3H/HeN. Results were expressed as the mean of 6 ears ± SD. There are significant differences between the positive control (immunized and challenged) and other groups: *P. binghamiae* i.p., heparin i.p., and *P. binghamiae* oral 2 days after challenge (Student's *t*-test, P<0.001). No challenge: immunized but not challenged with antigen.

The DTH response greater in magnitude and lasted longer in IL-5 transgenic mice may be explained by the presence of a higher number of eosinophils proliferated selectively in response to IL-5. Migration of eosinophils to the site of inflammation is one of the features of a DTH response. We found that eosinophils are increased at the site of inflammation in the DTH response against PCl in IL-5 transgenic mice. We examined whether the migration of eosinophils to the site of inflammation was suppressed by administrating algae polysaccharides. We measured the numbers of eosinophils in the ear section of transgenic mice and compared them between the non-treated group and polysaccharides-treated groups. *P. binghamiae* polysaccharides in either route of administration, i.p. or oral, significantly decreased the number of eosinophils at the site of inflammation 24 and 48 hours after antigen challenge (Fig. 3D, data not shown). Alginic acid suppressed slightly, but significantly, the number of eosinophils after 24 hours but not after 48 hours (Fig. 3D, data not shown). In contrast, fucoidan did not inhibit the number of eosinophils migrated to the site of inflammation 24 hours after the antigen challenge (Fig. 3D). The typical ear sections stained with hematoxylin and eosin are presented in Fig. 3A, B, and C. Cells with reddish-purple granules stained with eosin are eosinophils. Eosinophils were not observed without challenge with PCl (Fig. 3A and D). They were increased in number after the challenge of immunized mice (Fig. 3B and D) and decreased after treatment with *P. binghamiae* polysaccharides (Fig. 3C and D).

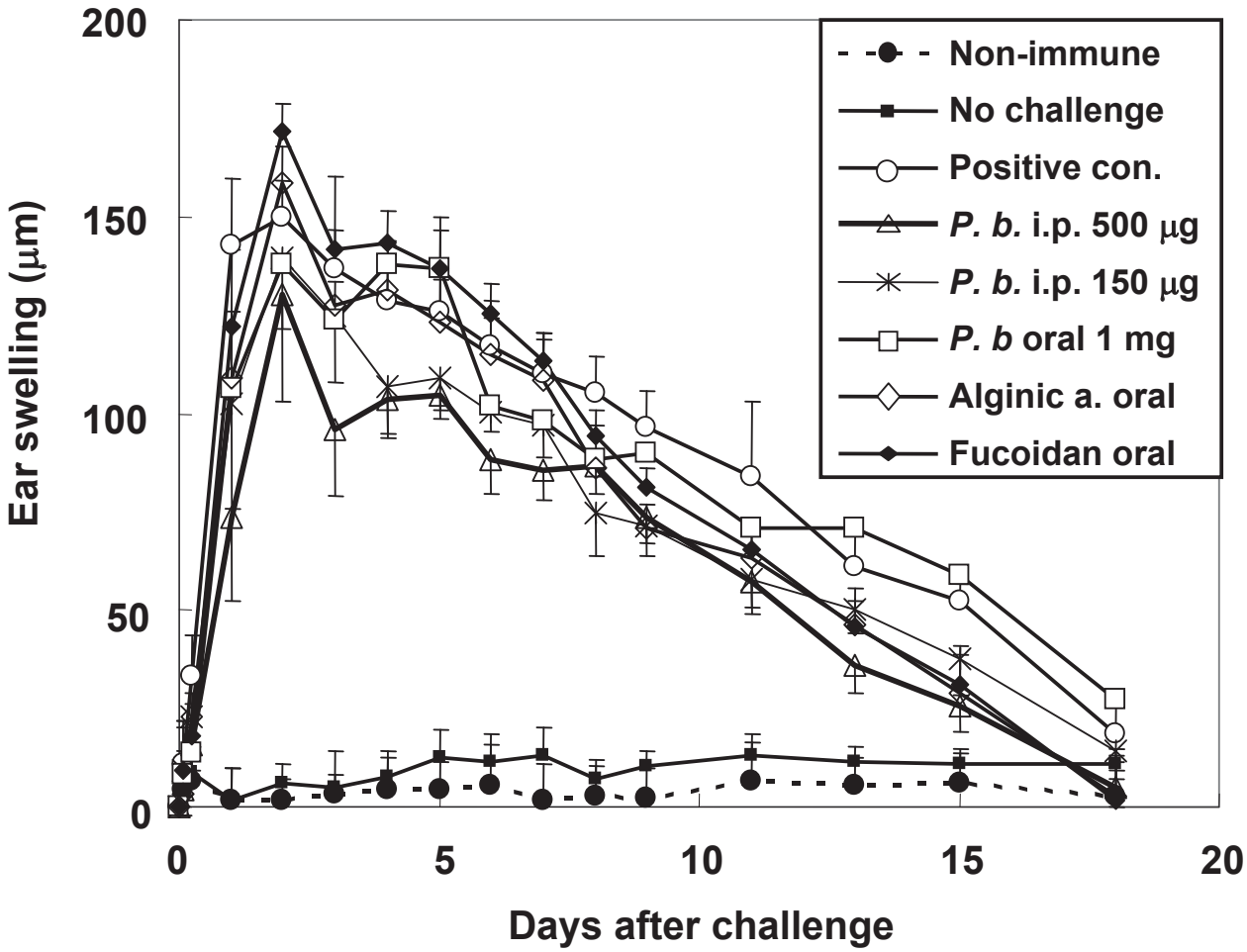

Figure 2. Suppression of DTH responses by algae polysaccharides in IL-5 transgenic mice.
DTH response was elicited against PCl as described in the section on materials and methods.
Reagents used to control the DTH response were *P. binghamiae* polysaccharides, alginic acid, and fucoidan. *P. binghamiae* polysaccharides were administered intraperitoneally (i.p.) at a dose of either 150 μg or 500 μg in 0.1 ml saline into each mouse 3 hours before antigen challenge. Oral: reagents were administered orally 1 mg/mouse every other day for two weeks after immunization until the antigen challenge. *P. b.: Petalonia binghamiae*. Results were expressed as the mean of 8 ears ± SD. There are significant differences between the positive control (immunized and challenged) and other groups: *P. binghamiae* i.p. 500 μg, *P. binghamiae* 150 μg., and *P. binghamiae* 1 mg oral at 2 days after challenge (Student's *t*-test, $P<0.05$). Non–immune: not immunized and not challenged with antigen. No challenge: immunized but not challenged with antigen. Positive control: immunized and challenged with antigen.

4. Discussion

Algae are eaten as health food in many Asian countries. However, the precise effects of algae on health have not been studied thoroughly. At first, we compared the effects of polysaccharides from *P. binghamiae* and *S. pacifica* with that of commercially available polysaccharides such as fucoidan or alginic acid in terms of NF-ϰB indicing ability in response to TLR 2 and TLR4 (Tominaga *et al.,* 2010). Both *P. binghamiae* polysaccharides and *S. pacifica* complex polysaccharides share special characteristics: namely the weak and equivalent responsiveness to TLR2 and TLR4. In contrast, fucoidan, alginic acid, λ-carrageenan, *E. coli* LPS induced NF-ϰB mainly in response to TLR4 in the assay using Golenbock's cells (Delude *et al.,* 1998, Medvedev *et al.,* 2001, Tominaga *et al.,* 2010, and our unpublished results). Signaling through both TLR4 and TLR2 may cause the different activity compared with signaling through TLR4 alone. Alginic acid suppressed the tumor (hepatocellular carcinoma MH134) growth both in C3H/HeJ and C3H/HeN (Tominaga *et al.,* 2010). On the other hand, *P. binghamiae* polysaccharides, fucoidan, and *S. pacifica* complex polysaccharides suppressed the tumor growth in C3H/HeN but not in C3H/HeJ (Tominaga *et al.,* 2010). It is speculated that sulfate containing polysaccharides may be related to the antitumor activity. However, another sulfate containing λ-carrageenan has a similar tendency with alginic acid in terms of antitumor activity using MH134 (our unpublished results). Further structural analysis is necessary to draw a conclusion.

In DTH reactions, both IFN-γ and IL-17 are

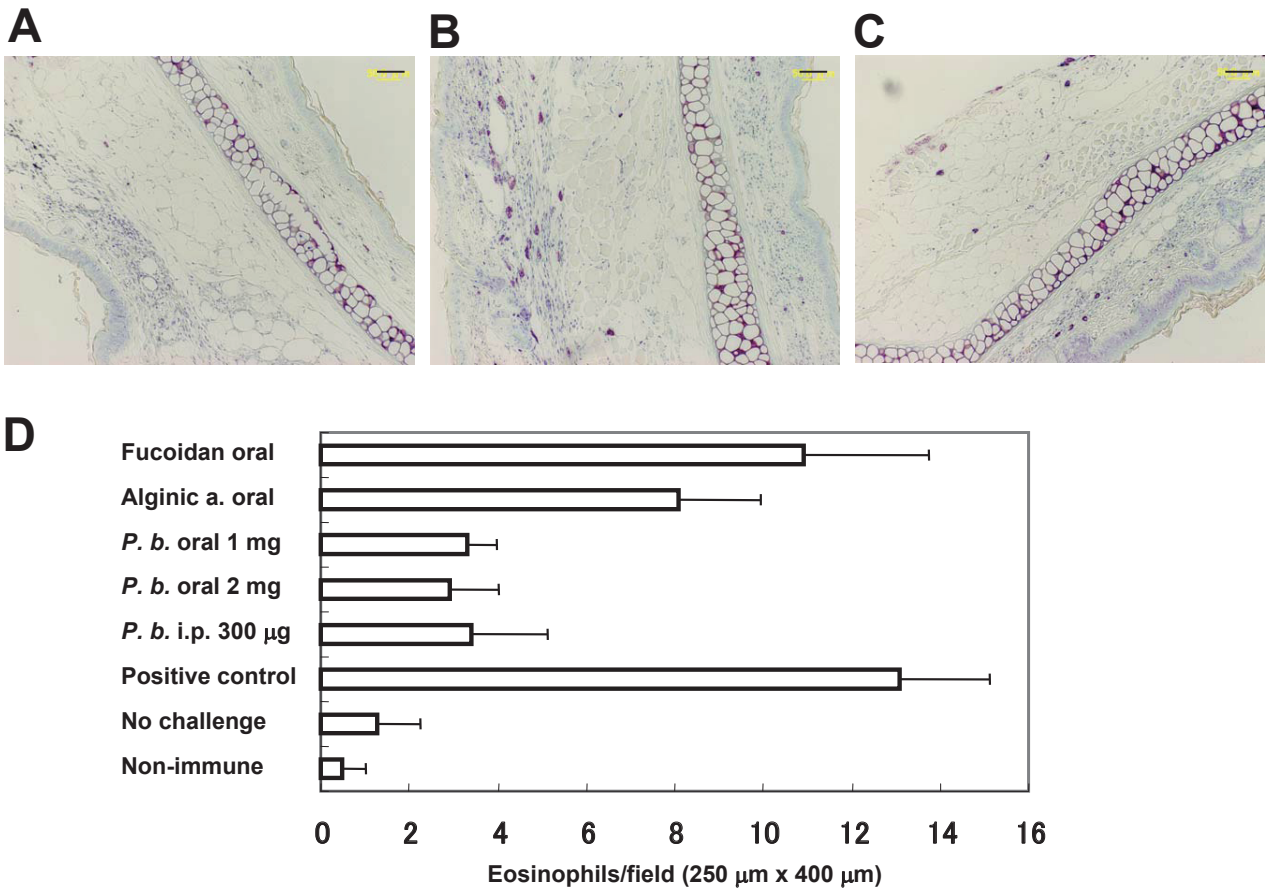

Figure 3. Effects of algae on the number of eosinophils migrated to the site of inflammation.
DTH reaction was elicited against PCl as described in the section on materials and methods. One day after challenge, ears were fixed with 4% paraformaldehyde in a 0.1 M Phosphate buffer, pH7.2 and tissue sections were stained with hematoxylin and eosin. Eosinophils were counted in an area of each ear section. Typical examples are shown in **Panels A, B, C**.
A: Section of an ear from an IL-5 transgenic mouse without antigen challenge. **B**: Section of an ear from an IL-5 transgenic mouse immunized and challenged with PCl (positive control). Eosin-stained eosinophils were accumulated between skin and muscle. **C**: Section of an ear from an IL-5 transgenic mouse immunized and challenged with PCl and administered with *P. binghamiae* polysaccharide i.p. **D**: Results were expressed as the average number of eosinophils of 10 fields (each field is 250 μm X 400 μm) ± SD. There are significant differences the between positive control and other groups: alginic acid, *P. binghamiae* 1 mg oral, *P. binghamiae* 2 mg oral, and *P. binghamiae* i.p. (300 μg/mouse) (Student's *t*-test. P< 0.001). Bars, 50 μm(A - C)

involved in inflammation in both C3H/HeN and C3H/HeJ (Tominaga *et al.,* 2010). Serum levels of IL-17 were strongly suppressed in the group of i.p. administration of *P. binghamiae* polysaccharides. However, the production of IL-17 was not suppressed significantly when *P. binghamiae* polysaccharides were administered orally. Although down-regulation of IL-17 is effective in reducing the DTH response, administration of neutralizing antibodies against IL-17 only partially suppressed the DTH response against PCl (Tominaga *et al.,* 2010). These results suggest that the down-regulation of IL-17 is partially effective in ameliorating the disease condition of DTH.

Serum levels of IL-17 were elevated when tumors (hepatocellular carcinoma, MH134) were growing and were down-regulated when tumor growth was suppressed weakly by polysaccharides (Table. 1). There are two types of polysaccharides relating to tumor suppression. One is alginic acid, which suppresses the growth of MH134 in both TLR4 mutant C3H/HeJ and wild type C3H/HeN. *P. binghamiae* polysaccharides, fucoidan and complex polysaccharides (lipopolysaccharide fraction) of *S. pacifica* suppressed the tumor growth only in C3H/HeN but not in C3H/HeJ, suggesting that the signal that suppresses the tumor growth is mediated through TLR4. Interestingly, these three polysaccharides stimulate the production of IFN-γ and enhance the tumor growth in C3H/HeJ (Table 1). Production of IFN-γ is reported to be effective in suppressing tumor growth (Shankaran *et al.,* 2001). *P. binghamiae* polysaccharides, fucoidan, and in particular complex polysaccharides of *S. pacifica* induced the production of IFN-γ in both C3H/HeJ and C3H/HeN mice. Alginic acid, on the other hand, did not induce the production of IFN-γ, though it suppressed tumor growth efficiently (Table 1). These results suggest that IFN-γ is not always protective against tumor growth.

In terms of anti-DTH reaction, *P. binghamiae* polysaccharides showed the highest suppressive activity. Alginic acid did not suppress the DTH response 48 hours after antigen challenge (Fig. 1 and Fig. 2). In contrast, heparin, which contains high amounts of sulfate and does not induce NF-κB in response to either TLR2 or TLR4, suppressed the DTH as strongly as *P. binghamiae* polysaccharides (Fig. 1).

Migration of eosinophils is one of the factors that stimulate inflammation. In the IL-5 transgenic mice, we could observe the increased number of eosinophils at the site of inflammation in the DTH response against PCl (Fig. 3B). Since *P. binghamiae* polysaccharides suppressed the number of eosinophils migrated to the site of inflammation much more efficiently than alginic acid, sulfate moiety might be involved in the inhibition of the migration of eosinophils. Recently, Mitoma *et al.* (2007) concluded that 6-sulfo sialyl Lewis X on N-glycan has a critical function as an L-selectin ligand and is essential for lymphocyte trafficking in normal and disease conditions including contact hypersensitivity against dinitrofluorobenze.

Although it is quite possible that *P. binghamiae* polysaccharides inhibit the interaction between L-selectin on eosinophils and 6-sulfo sialyl Lewis X on N-glycans of high endothelial venules, the structural difference between *P. binghamiae* polysaccharides and fucoidan both of which seem to contain sulfate groups in polysaccharide chains remains to be clarified.

In conclusion, some sea algae polysaccharides such as those from *P. binghamiae* are quite useful in manipulating the immune response to ameliorate the allergic inflammation or to suppress the tumor growth. Some algae may be used as an alternative medicine to regulate immune responses.

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Table 1. Effects of polysaccharides on the growth of hepatocellular carcinoma MH134 and serum levels of IFN-γ and IL-17.

	C3H/HeN			C3H/HeJ		
	Growth	IFN-γ	IL-17	Growth	IFN-γ	IL-17
No tumor	0	+	++	0	++	++
Saline	++++++	+	++++	++++++	±	+++
Alginic a., i.p.	++	±	++	++	++	++
P. binghamiae, i.p.	+++	++	+++	+++++++	++++++	+++
Fucoidan, i.p.	++++	++	+++	++++++++	++++++	++++
S. pacifica, i.p.	++++	++++++	+++	++++++++	++++++	++++

The suppression of the tumor growth was evaluated 20 days after one million MH134 tumor cells were inoculated on the back of each mouse (Tominaga *et al.,* 2010 and our unpublished results).

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