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Suppression of Th2 immune responses by the sulfated polysaccharide from *Porphyra haitanensis* in tropomyosin-sensitized mice

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ABSTRACT

The sulfated polysaccharide from *Porphyra* was hypothesized to exhibit immunoregulatory, anti-tumor and anti-inflammatory activity, but its anti-allergic activity is not fully understood. Therefore, the aim of this study was to isolate sulfated polysaccharide from *Porphyra haitanensis* (PHPS) and investigate its anti-allergic potential using a tropomyosin (TM)-induced mouse allergy model. Intraperitoneal injection of PHPS suppressed the allergic reaction by modulating serum IgE, IgG1 and IgG2a levels in mice. In particular, when PHPS was injected prior to the first immunization with TM, the IgE level decreased by 34.2% compared with the control (PBS) group. Oral therapeutic administration of PHPS to TM-sensitized mice decreased histamine release and repaired the pathology in the jejunum of the small intestine. *In vitro*, the mRNA expressions of the TM-induced Th2 cytokines (interleukin-4 (IL-4), IL-5 and IL-13) in splenic lymphocytes were reduced by PHPS; however, the expression of Th1 and regulatory cytokines (interferon gamma (IFN-γ) and IL-10) were up-regulated in PHPS-treated splenic lymphocytes. In the splenic lymphocyte supernatant, the IL-4, IL-13 and IFN-γ levels were also regulated by PHPS. Moreover, PHPS induced IFN-γ secretion via the Jun N-terminal kinase (JNK) and Janus kinase 2 (JAK2) signaling pathways. Therefore, these results suggest that PHPS suppresses the TM-induced allergic reaction, possibly by modulating the imbalance of the Th1/Th2 immune response.

1. Introduction

Food allergy is an abnormal immunologic disease affecting nearly 8% of children and 5% of adults, with growing evidence of an increase in prevalence [1]. Milk, eggs, peanuts and crustaceans are common allergenic foods, and crustaceans are one of the eight major allergenic foods [2]. In the USA, shellfish is the number one cause of IgE-mediated food allergy in persons older than 6 years [3]. Shellfish allergy in the Asia-Pacific region ranks among the highest in the world and is the most common cause of food-induced anaphylaxis [4]. In China, 16.7% of the rural population is sensitized to shellfish [5]. The major allergen in the shellfish has been identified as the muscle protein tropomyosin (TM) [6]. With the rapid growth of crab consumption, researchers all over the world are paying much more attention to the crab allergens. Our laboratory has purified and identified the TM moiety in *Eriocheir sinensis*[7], and we have proved that TM is an allergen in crab through digestion experiments and a mouse model [8,9].

Food allergy is a representative of IgE-mediated type I hypersensitivity and is characterized by the development and activation of T helper 2 (Th2) cells [1,10]. The differentiation of Th2 cells leads to the production of IL-4, IL-5 and IL-13, which results in the binding of allergens to mast cells and basophiles [11,12]. When Th1 cells produce IFN-γ, the allergy symptoms may be relieved since IFN-γ can suppress Th2 differentiation and proliferation [12,13]. Therefore, an allergen-induced Th1/Th2 imbalance leads to allergic inflammation.

Porphyra has been used as a drug in traditional Chinese medicine to relieve sore throat, fever and oedema [14]. *Porphyra* is a red alga and is widely consumed in Asia. In Japan, *Porphyra* is usually used to prepare nori, which is a component of the traditional Japanese food, sushi. However, most investigations to date have focused on *Porphyra yezoensis*, a species widely distributed in East Asia. In China, *Porphyra haitanensis* is a top-grade *Porphyra*, and it has been used as an imperial tribute to royalty since the Song dynasty. We investigated *P. haitanensis*, which is an important economic alga in south China nowadays [15].

Polysaccharide accounts for approximately 25–40% of the total mass of dried *Porphyra*[16]. Sulfated polysaccharide from *P. haitanensis* (PHPS) comprises the hot-water soluble portion of the cell wall in *Porphyra*. PHPS is composed of sulfated polygalactans consisting of galactose and 3,6-anhydrogalactose units, which are sometimes substituted with galactose-6-sulfate and 6-*O*-methyl-galactose [17]. During the

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past few years, many studies have investigated the immune-modulating activities of the polysaccharide in *Porphyra*. Yoshizawa et al.[18,19] have shown that the polysaccharides in *Porphyra yezoensis* possess macrophage-stimulating activity *in vivo* and *in vitro*. Ishihara et al.[20] have confirmed that the sulfated polysaccharides in nori had the ability to suppress the contact hypersensitivity reaction by suppressing IgE and promoting IFN-γ in serum. Bhatia et al.[21] have found that the sulfated polysaccharides in *Porphyra vietnamenis* possess potential immunomodulatory activity and have therapeutic potential for the prevention of autoimmune diseases.

The aim of this study was to isolate sulfated polysaccharide from *P. haitanensis* and explore its anti-allergic activity with respect to the specific immune responses of mice to the allergen TM. To explore our hypothesis, we investigated the *in vivo* therapeutic potential of intraperitoneal injection or oral administration of PHPS on the TM-induced Th2 response in the mouse model. Moreover, we analyzed cytokine production by spleen lymphocytes isolated from TM-sensitized mice that were re-stimulated *in vitro* by the allergen in the presence or absence of PHPS.

2. Materials and methods

2.1. Animals

Specific pathogen-free (SPF) 6-week-old female BALB/c mice were purchased from the Shanghai laboratory animal center of the Chinese Academy of Sciences (Shanghai, China). Mice were housed in an SPF environment maintained at 22 ± 1 °C with a relative humidity of 55 ± 10%. All protocols were approved by the Fisheries College, Jimei University.

Scylla paramamosain was purchased from a local market in Xiamen (China) and used for the purification of TM.

2.2. Purification of PHPS

P. haitanensis that grew in Dadeng Island (Xiamen, China) were purchased from a local market in Xiamen. PHPS was extracted and purified according to the procedures of Zhou et al. [22]and Zhang et al. [23]. Dried material (25 g) was crushed and boiled in distilled water (1 L) for 4 h. The mixture was centrifuged. The volume of the supernatant was reduced to 1/3 of the initial volume at 65 °C by rotary evaporation. The crude polysaccharides were precipitated by adding 4 volumes of 95% ethanol. The crude polysaccharides were dissolved in distilled water and applied to a column (3.5 × 50 cm) of DEAE-cellulose 52 (Whatman, New York, USA) that had been equilibrated with distilled water. Distilled water was added to remove the components that did not bind to the column, such as the neutral polysaccharides, proteins, inorganic substances and pigments. The absorbed materials were eluted with a 2.0 M NaCl solution. The carbohydrate content of the eluate was detected by the anthrone-sulfate acid method [24]. The eluate containing carbohydrate was collected and dialyzed to obtain the PHPS. The lipopolysaccharide (LPS) was removed from PHPS with Detoxi-Gel Endotoxin Removing Gel (Pierce, Rockford, USA). The LPS content of PHPS was measured and found to be < 0.03 EU/mg as measured by the Limulus amebocyte lysate assay (Associates of Cape Cod, New York, USA).

2.3. Chemical analysis of PHPS

The carbohydrate content, 3,6-anhydrogalactose (3,6-AG), sulfate, uronic acid and protein contents were measured by the anthrone-sulfate method [24], ester sulfate-methoxyl method [25], barium chloride-gelatin method [26], sulfate-carbazole method [27] and the Lowry method [28], respectively, using galactose, fructose, potassium sulfate, glucuronic acid and bovine serum albumin (BSA) as standards, respectively. To determine the monosaccharide composition of PHPS, the PHPS acid hydrolysate was applied to an ion chromatography (IC) (ICS-3000, Dionex, Sunnyvale, CA, USA) column (CarboPac PA10 [4 × 250 mm]) and separated using 0.02 M NaOH at a flow rate of 0.6 mL/min.

2.4. Immunization of mice

TM was extracted and purified from *S. paramamosain* as previously described [7]. In the preliminary experiments, the effect of PHPS on the mice was investigated. The mice of PHPS group were intraperitoneally injected with PHPS (100 μg/mouse) three times every week from days 0 to 14 and bled on day 15. After the intraperitoneally injection with PHPS, the mice were fed with PHPS (5 mg/mouse) on days 28, 30, 32 and 34. The fecal samples were collected to evaluate the histamine levels 1 h after treatment. The same volume of PBS was regarded as the unimmunized group (PBS group).

Mice were immunized intraperitoneally with 0.15 mL phosphate buffer solution (PBS) containing the purified TM (100 μg/mouse) and alum adjuvant (Pierce, Rockford, USA) on days 0 and 14 (Fig. 1A). The negative control mice were given intraperitoneal injection of 0.15 mL PBS and regarded as the unimmunized group (PBS group). During the course of TM sensitization, mice were intraperitoneally injected with 100 μg/mouse PHPS from 7 days before (regarded as the PHPS preventive group) or 1 day after the first immunization (regarded as the PHPS treatment group) three times every week. The TM-sensitized mice injected with PBS instead of PHPS were referred to as the positive control (TM group) in which an allergic reaction was maintained. These mice were bled on day 15. The blood was centrifuged at 4000*g* for 10 min at 4 °C. The serum samples were collected and stored at -80 °C for future assay.

From day 28, each mouse from the TM group and PHPS preventive group was challenged 10 times at 2 to 3day intervals by gavages of 10 mg TM in a final volume of 200 μL PBS (Fig. 1A) according to the procedure of Costa et al. [29]. Before each gavage, mice were deprived of food for 3 h, and 1 h before, each TM-challenged mouse from the PHPS preventive group was treated with 5 mg PHPS/mouse. On day 49, mice were re-challenged as described above, and fecal samples were collected to evaluate the histamine levels 1 h after treatment. On day 50, mice were killed to collect the jejunum.

2.5. Measurement of the TM-specific IgE, IgG1 and IgG2a in sera

The TM-specific IgE level was measured by indirect ELISA [30]. Briefly, 96-well plates were coated with 100 μL purified TM solution and maintained overnight at 4 °C. The wells were washed with 300 μL wash buffer (20 mM Tris, 0.145 M NaCl, 0.05% Tween 20, pH 7.5) and then blocked with 300 μL blocking solution (5% skim milk in PBS) at 37 °C for 2 h. After washing, 100 μL diluted sera (1:6 dilution) were added to each well, and the wells were incubated overnight at 4 °C. After the wells were washed, 100 μL of 1:1000 dilution for goat anti-mouse IgE-HRP secondary antibody (Southern Biotech, Birmingham, USA), 1:5000 dilution for goat anti-mouse IgG1 heavy chain (HRP) secondary antibody or 1:5000 dilution for goat polyclonal secondary antibody to mouse IgG2a-heavy chain (HRP) (Abcam, Cambridge, UK) was added to the wells, and the wells were incubated at 37 °C for 2 h. The wells were then washed before the addition of 100 μL TMB substrate solution (Tiangen, Beijing, China). The wells were incubated at 37 °C for 20 min. The color reactions were stopped with 100 μL stop solution (2 M H_2SO_4). The absorbance at 450 nm was read with an ELISA plate reader (Bench mark 96, Bio-Rad, Hercules, CA, USA). The TM-specific serum IgE level was also detected by Western blotting analysis according to the method of Huang et al. [8].

2.6. Measurement of the histamine in fecal extracts

Histamine in fecal samples was measured using high-performance liquid chromatography (HPLC) according to the procedure of Hu et al.