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NOTE

Immunology

Oral λ-carrageenan intake alleviates skin symptoms in a hapten induced atopic dermatitis-like model

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ABSTRACT. Lambda carrageenan is a widely used food additive. It has been shown that its oral intake induces suppression of T cell proliferation and antibody-mediated and cell-mediated immune response in experimental animals. In this study, we estimated the effect of oral ingestion of 0.001% λ-carrageenan on trinitrochlorobenzen-induced atopic dermatitis model mouse. Oral carrageenan ingestion alleviated ear swelling of hapten challenged mice and significantly suppressed mast cell hyperplasia in the topical skin. Serological analysis revealed that the treatment suppressed total IgE and antigen-specific IgG, and also suppressed both allergy driving cytokine interleukin-4 and counter-acting cytokine interferon-γ levels. It is suggested that the oral ingestion of λ−carrageenan may suppress the immunological response to the allergen and might be useful to treat atopic dermatitis.

KEY WORDS: atopic dermatitis, carrageenan, oral

Lambda carrageenan is a sulfated poly galactan extracted from red edible algae. It is widely used as a food and pharmaceutical additive and formula for thickening, gelling and stabilizing agent, and is recognized as safe as Drug Administration as Joint FAO/WHO expert committee on food additives held in 2014 stated that "the use of carrageenan in infant formula or formula for special medical purposes at concentrations up to 1,000 mg/*l* is not of concern" [14].

Lambda carrageenan also has been known to be an inflammatory reagent when injected parenterally. Its property has been commonly used in experimental inflammation animal models such as footpad edema and pleurisy [9, 10]. In contrast, orally ingested λ-carrageenan has been shown to suppress antibody response and T-cell proliferation [4, 16].

Atopic dermatitis (AD) is a chronically relapsing inflammatory skin disease characterized by skin lesions with intense pruritic, erythematous papules associated with scratch, vesiculations, and serous exudate [6, 15]. In the AD patients, the number of mast cells and eosinophils increased in the lesional skin tissues [5]. It has been shown that serum IgE and IgG levels to environmental antigens increased in AD patients [3, 13], and the role of cytokines such as interleukin-4 (IL-4) and interferon-γ (IFN-γ) have been reported [12, 17].

To study the pathogenesis of AD, several animal models have been established, and a hapten induced mouse model is widely used one [1, 7]. In the model, the abdominal skin of BALB/c mice was sensitized with trinitrochlorobenzene (TNCB), and their ears were repeatedly challenged with the same hapten. Within a few weeks, they develop AD-like skin lesions associated with eosinophilic inflammation and an increase in the number of mast cells and total IgE, which are hallmarks of AD.

In this study, we investigated the effect of oral ingestion in the hapten induced atopic dermatitis model. Oral ingestion of 0.001% λ-carrageenan in drinking water alleviated skin swelling in the model, and it was associated with suppressed antibody and cytokine levels in the serum, suggesting the potential of λ-carrageenan as a therapeutic agent to ameliorate AD.

All animal experiments were performed under the protocol approved by the Animal Experiment Committee of Osaka Prefecture University and following the ethical guidelines of the institute. Eight-week-old female BALB/c mice (Oriental Yeast Co., Ltd., Tokyo, Japan) were given freshwater (carrageenan-nontreated mice) or water containing 0.001% λ-carrageenan (carrageenan-treated mice) *ad libitum* throughout the experimental period from day-10. To induce hapten induced AD-like symptoms, mice were sensitized with 5% TNCB (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) in acetone to the abdomen on day −4 and −3 and subsequently challenged with the application of 1% TNCB on the ear lobe from day 0 to day 22 every two days. The thickness of the TNCB-challenged ear lobe was measured using a dial thickness gauge every four days from day 0. Mice were euthanized with heart puncture under the deep anesthesia to collect serum and ear lobe tissue samples on day 24. Sera were preserved under −30°C until analysis. Three μm section of formalin-fixed and paraffin-embedded tissue samples were stained with hematoxylin and

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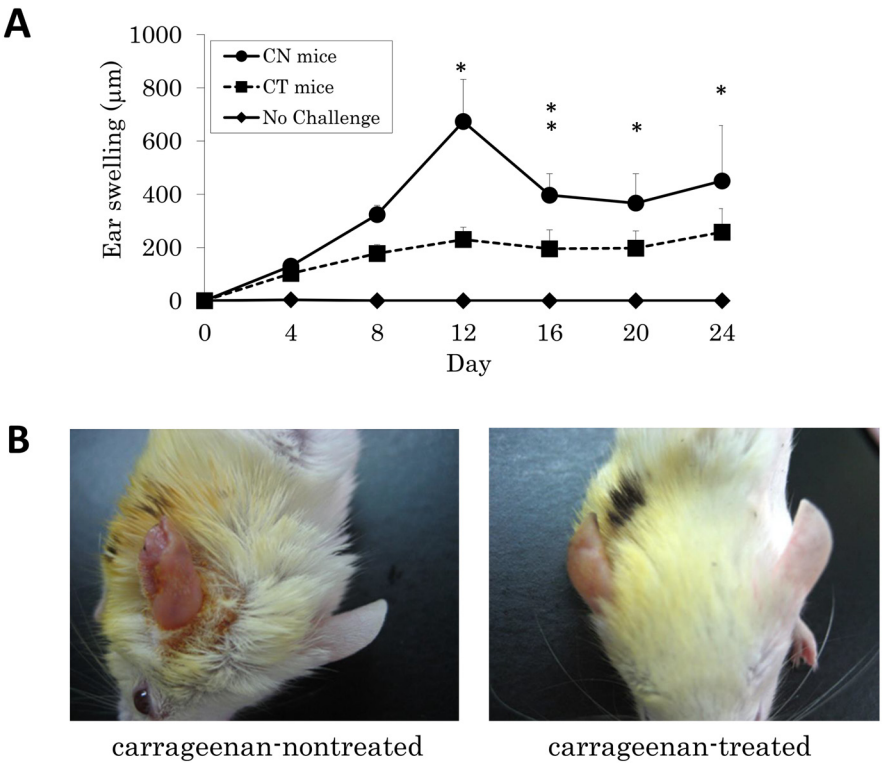

Fig. 1. Ear swelling in trinitrochlorobenzen-induced atopic dermatitis model. (A) Change in ear thickness during repeated TNCB challenge. (B) Ear swelling of TNCB-challenged mouse on day 24. **P*<0.05, ***P*<0.01 compared between carrageenan treated (CT) and non-treated (CN) groups by Tukey-Kramer multiple-comparisons test.

eosin (HE) as well as toluidin blue to detect mast cells. TNCB-specific IgG titer in the sera was measured by ELISA [11]. TNCB was conjugated to bovine serum albumin (BSA) by the overnight incubation of 400 mg TNCB with 100 mg of BSA in 8 m*l* of 0.2 M $NaHCO_3$. ELISA plates were coated with TNCB-BSA, blocked with 1% ovalbumin in PBS (OVA-PBS), and reacted with serum samples serially diluted with OVA-PBS for one hour. The plates were washed with 0.5% Tween 20 in PBS (wash buffer), then reacted with horseradish peroxidase-conjugated goat-anti mouse IgG for one hour. The plates were again washed with wash buffer, and 100 μl of substrate solution (0.4 mg/*l* *o*-phenylenediamine in PBS) was added to each well. After 5 min, reaction was stopped by the addition of 100 μl of 2N H_2SO_4 and the optical densities at 490 nm were read using an ELISA plate reader (BioRad Laboratories, Hercules, CA, USA). The inverse of the greatest dilution that gives a positive result was defined as the titer of the serum. Total IgE concentration was estimated by mouse total IgE measuring kit (Morinaga, Yokohama, Japan). The concentration of IL-4 and IFN-γ were measured by Quantikine ELISA kits for mouse IL-4 and mouse IFN-γ respectively (R&D systems, Minneapolis, MN, USA). All data are represented as the mean values with error bars representing standard error. The statistical significance was evaluated by Student's *t*-test or Tukey-Kramer multiple-comparisons test. Values of $P<0.05$ were considered statistically significant.

As reported previously, repeated application of TNCB after sensitization induced skin lesions accompanied by swelling of ear lobes. As shown in Fig. 1, the thickness of the ear lobe increased from day 4 by repeated TNCB challenge in the carrageenan-nontreated mice (CN mice), but it was significantly alleviated in carrageenan-treated mice (CT mice) mice from day 8 to the end of the experimental period (Fig. 1A and 1B). Microscopical analysis of HE stained tissue section of challenged ear lobe showed hyperplasia of epithelial layer and infusion of eosinophilic inflammatory cells in both CN and CT mice on day 24, and a significant difference was not observed between two groups (Data not shown). However, when we analyzed mast cell numbers in the tissue by toluidin blue staining analysis (Fig. 2A), a significant number of mast cells were observed in the epidermis area in CN mice, but it was significantly decreased in CT mice (Fig. 2B).

To evaluate the effect of carrageenan ingestion on antibody production, we measured the titer of total TNCB-specific IgG and concentration of total IgE in sera on day 24 (Fig. 3). TNCB-specific IgG titer was slightly but significantly lower in CT mice than CN mice (Fig. 3A), and total IgE concentration was also significantly suppressed in CT mice (Fig. 3B). When we estimated serum IL-4 and IFN-γ in serum (Fig. 4), the concentration of both cytokines increased after sensitization and challenge by TNCB, but those concentrations were significantly lower in CT mice than in CN mice.

In this study, we evaluated the effect of orally-ingested carrageenan on the hapten induced AD model mouse. Carrageenan intake significantly suppressed skin swelling and mast cell hyperplasia in the lesion, which are hallmarks of AD [5]. Mast cells have been shown to increase in the lesional skin of AD and to release mediators such as histamine, prostaglandins, leukotrienes, tryptase;

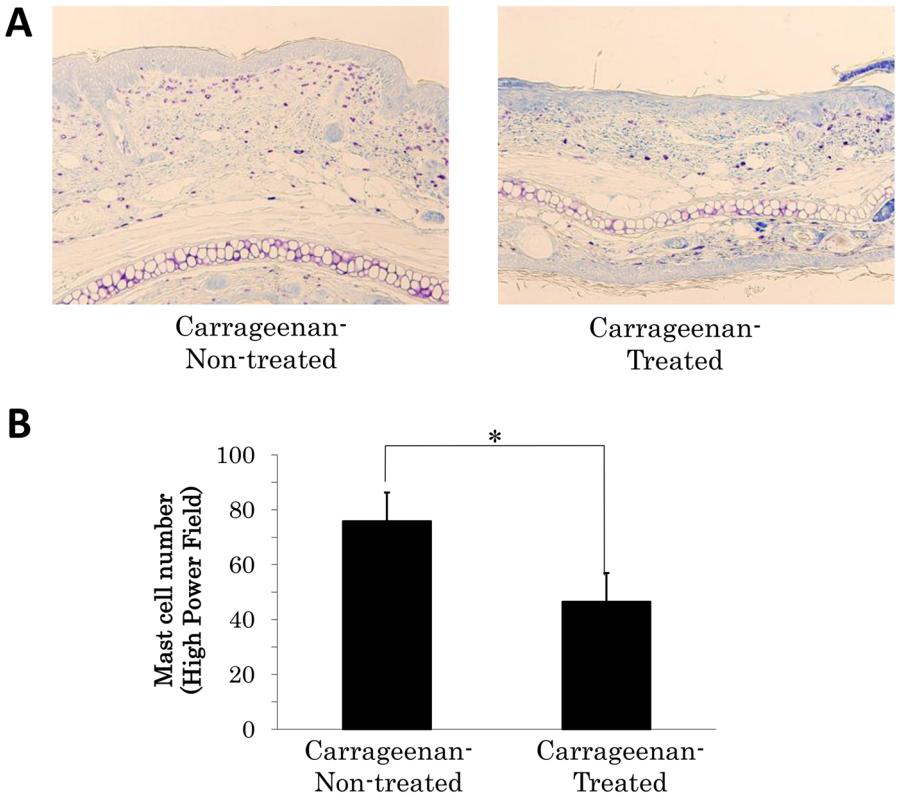

Fig. 2. Mastocytosis after trinitrochlorobenzen challenge on day 24. (A) Toluidine blue-stained sections of the TNCB-challenged ear. Magnitude, ×100. (B) Numbers of mast cells in randomly selected three high-power field at a magnification of ×400. *P<0.05 by Student's t-test.

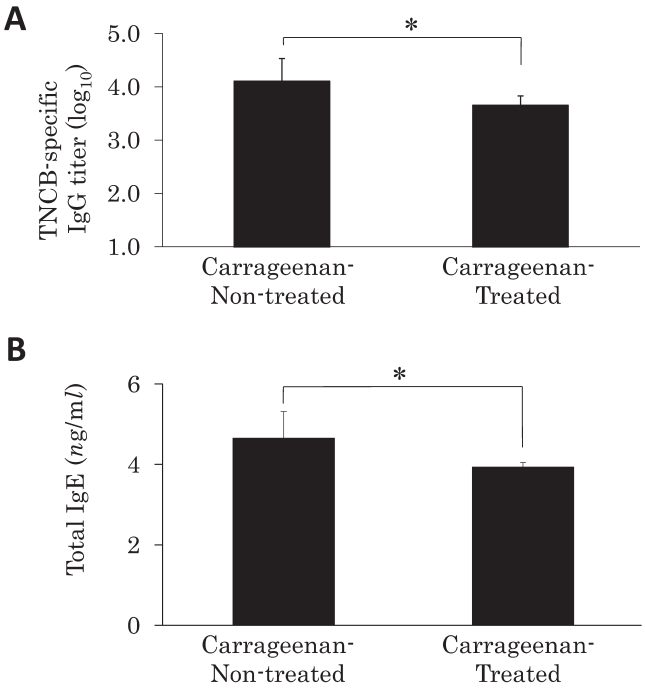

Fig. 3. Serological analysis of trinitrochlorobenzen-challenged mice on day 24. Antigen-specific IgG titer (A) and total IgE (B) concentration in the serum analyzed by ELISA. *P<0.05 by Student's t-test.

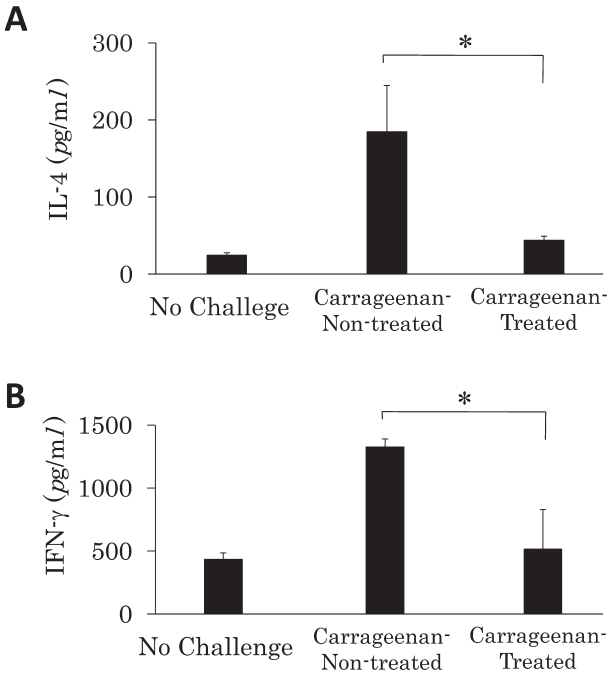

Fig. 4. Cytokine concentrations in the serum on day 24. Interleukin-4 (A) and interferon-γ concentration in the serum analyzed by ELISA. *P<0.05 by Tukey-Kramer multiple-comparisons test.

thus, they have been thought to contribute to inflammatory reactions [8]. The decrease in mast cells in the lesional skin of CT mice seems to reflect the attenuation of allergic inflammation in CT mice.

Total IgE and antigen-specific IgG were suppressed by oral carrageenan treatment. IgE causes not only immediate allergic reaction but also late phase one, which leads to chronic allergic inflammations [1]. Thus, it seems that the suppression of IgE in the serum contributed to the alleviation of TNCB-induced symptoms.

Orally-ingested carrageenan has been shown to have immuno-modulatory activity, such as immunosuppression, induction of oral tolerance, and modulation of cytokine balance. Tsuji *et al*. showed that orally administrated carrageenan induces IFN-γ secretion from splenocytes *in vivo* and suppresses IgE sensitization to parenterally injected allergen when orally administrated [16]. Although the results seem contradictory to ours, there is a difference in the carrageenan administration method; they administrate carrageenan (0.001%) once a day by oral gavage while we gave the same concentration of carrageenan containing water at *ab lib*, this may lead to a higher concentration of λ-carrageenan in the mouse blood in our experiment. They also showed low concentration (4–20 μg/m*l*) of carrageenan promoted IFN-γ and IL-10 secretion and suppressed IL-4 from mitogen-stimulated splenocytes *in vitro*, while it suppresses IFN-γ and IL-4 and enhances IL-10 at higher concentration (100–500 μg/m*l*). As IL-10 is known as an immunosuppressive cytokine, increased secretion of the cytokine might have led to the suppression of IL-4 and IFN-γ, resulted in the reduced induction of IgG, and IgE thus may alleviate AD-like symptoms of TNCB-treated mice.

It has been reported that the immunomodulatory activities of λ−carrageenan are toll-like receptor 4 (TLR4) dependent and independent. TLR4 is considered to mediate immunostimulatory response like the induction of inflammation and suppression of oral tolerance [16], thus the inhibition of IFN-γ, IL-4 and antibody production seems to be caused by unknown TLR4 independent mechanism. It has also been shown that oral ingestion of λ- carrageenan inhibited *in vitro* spleen T cell proliferation and it was mediated by the adherent macrophage-like cell [2]. Suppressive activity of λ-carrageenan seems to be attributed to these cells.

Our results show that orally-ingested carrageenan alleviates AD-like symptoms such as skin-swellings and mastocytoma in TNBC-induced AD model mouse, possibly by suppression of serum IL-4, IFN-γ, and total IgE. Thus, carrageenan may be useful for the prevention of atopic dermatitis.

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