

Oral Immunotherapy for Allergic Conjunctivitis Using Transgenic Rice Expressing Hypoallergenic Antigens

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Abstract: Pollinosis, or allergic conjunctivitis and rhinitis induced by pollen, is one of the most common diseases worldwide. In Japan, Japanese cedar (*Cryptomeria japonica*) pollinosis is a predominant allergic condition that affects more than one-third of all Japanese individuals. Pharmacological treatments of allergic conjunctivitis include administration of antiallergic eye drops containing an antihistamine or mast cell stabilizer. However, these topical treatments provide transient relief from symptoms. The only available curative treatment for allergic diseases is allergen-specific immunotherapy. Sublingual immunotherapy for pollinosis has been found to be effective for suppression of ocular and nasal symptoms, but patient compliance is low. Oral administration of staple foods engineered to express allergens is a possible means of delivering antigens for immunotherapy, and its convenience would be expected to improve compliance. With the aim of developing more convenient, effective, and safe immunotherapies for allergic diseases, we have generated rice-based edible vaccines expressing antigens derived from dust mites or pollen from Japanese cedar, birch, or ragweed. In this study, we summarize the results of our immunotherapy studies using transgenic rice. Oral immunotherapy with transgenic rice seeds containing hypoallergenic modified forms of Japanese cedar pollen antigens was effective for both preventing allergic conjunctivitis and suppressing established disease in mice. Oral administration of transgenic rice seeds is thus a promising

approach to immunotherapy for conjunctivitis and rhinitis induced by Japanese cedar pollen.

Key Words: allergic conjunctivitis, oral tolerance, transgenic rice, immunotherapy, pollinosis

(*Cornea* 2018;00:1–7)

Seasonal allergic conjunctivitis induced by pollen is a global clinical problem.^{1–3} Allergic conjunctivitis induced by ragweed pollen or birch pollen is common in the United States and Europe, whereas seasonal allergic conjunctivitis and rhinitis triggered by Japanese cedar (*Cryptomeria japonica*) pollen are considered national, increasingly prevalent afflictions in Japan, where they affect over one-third of the population.^{4,5}

Use of goggle-type eyeglasses, facemasks, and other barriers to allergens and topical pharmacological approaches are currently preferred for treatment of allergic conjunctivitis.⁶ Pharmacological treatments are not antigen specific and mainly comprise antiallergic eye drops containing an antihistamine or mast cell stabilizer (or both) and steroid eye drops. However, novel treatments are needed because these topical treatments provide only transient symptom relief and do not cure the underlying allergy. At present, allergen-specific immunotherapy is the only potentially curative treatment available for allergic diseases.

In this review, we describe the current and novel approaches to allergen-specific immunotherapy using the rice-based allergy vaccines that we have generated as possible curative treatments for allergic conjunctivitis.

ALLERGEN-SPECIFIC IMMUNOTHERAPY

Allergen-specific immunotherapy was first introduced in 1911 by Leonard Noon⁷ and initially comprised subcutaneous immunotherapy (SCIT). Noon showed that subcutaneous administration of a grass pollen extract suppressed immediate conjunctival sensitivity to pollen, as measured by a conjunctival antigen challenge test. Although SCIT is effective for treating allergic diseases including rhinoconjunctivitis,^{8,9} it requires frequent hospital visits for injections, involving both pain and inconvenience for patients. Furthermore, SCIT is occasionally associated with severe systemic side effects, such as anaphylaxis because it involves the use of natural allergen extracts. Consequently, alternative routes of antigen administration, including

Received for publication June 29, 2018; revision received July 23, 2018; accepted July 24, 2018.

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K. Fukuda has received funding from the Charitable Trust Fund for Ophthalmic Research in Commemoration of Santen Pharmaceutical's Founder and an honorarium and travel fees from Santen Pharmaceutical. K. Fukuda has also received grants from Otsuka Pharmaceutical Co., Ltd., Novartis Pharmaceuticals Japan (Novartis Pharma K.K.), and Alcon Japan, and payment for lectures from Alcon Japan Ltd., Kissei Pharmaceutical Co., Ltd., Torii Pharmaceutical Co., Ltd., Senju Pharmaceutical Co., Ltd., Santen Pharmaceutical Co., Ltd., Eisai Co., Ltd., Kowa Pharmaceutical Co., Ltd., and Otsuka Pharmaceutical Co., Ltd. F. Takaiwa and Y. Wakasa have received a grant from the Agri-Health Translational Research Projects of the Ministry of Agriculture, Forestry, and Fisheries of Japan. A. Fukushima has received a grant from JSPS KAKENHI (Grant Number: JP16K11324). The remaining authors have no funding or conflicts of interest to disclose.

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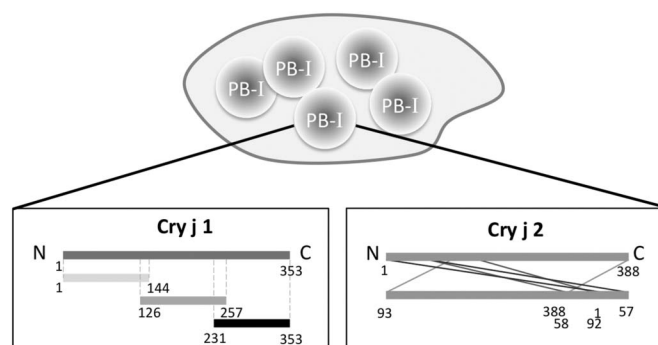


FIGURE 1. Expression of modified forms of the Japanese cedar pollen allergens Cry j 1 and Cry j 2 in a transgenic rice seed. Cry j 1 is modified by fragmentation and Cry j 2 is modified by molecular shuffling. PB-I, protein body-I.

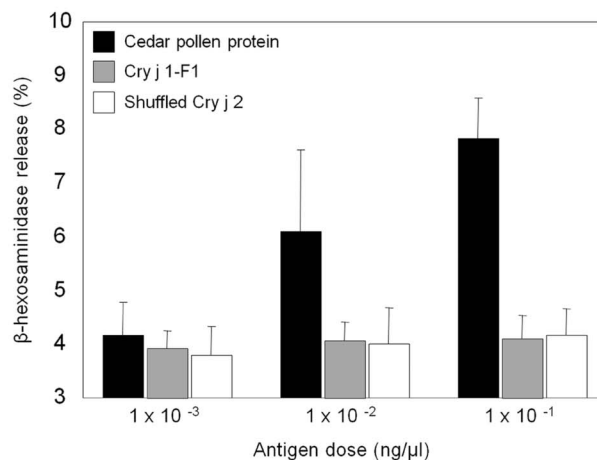
epicutaneous, intralymphatic, sublingual, and conjunctival applications, have been investigated.^{10–12}

Local conjunctival immunotherapy for allergic conjunctivitis has been investigated in animal models and patients. In a mouse model of allergic conjunctivitis, therapeutic antigen administration in eye drops significantly suppressed infiltration of inflammatory cells into the conjunctiva during the late phase but not the clinical symptoms in the early phase.¹³ In guinea pigs, topical exposure to antigens together with cholera toxin B, which acts as an adjuvant, suppressed the clinical and histological findings of experimental allergic conjunctivitis.¹⁴ However, the effectiveness of conjunctival immunotherapy in patients has remained unclear. Del Perete et al¹⁵ reported significant amelioration of subjective and objective symptoms, as well as fewer eosinophils on cytology, after treatment of patients with allergic conjunctivitis for 1 year. Núñez and Cuesta¹⁶ administered local conjunctival immunotherapy for 6 months in patients with allergic conjunctivitis induced by the dust mite *Dermatophagoides pteronyssinus*. A significant reduction in the symptom score evaluated by a conjunctival provocation test was obtained in patients receiving local immunotherapy but not in those treated with the placebo. A prospective, double-blind, randomized controlled trial of local conjunctival immunotherapy with a mixed *Dermatophagoides* extract for 6 months was performed in patients with allergic conjunctivitis.¹⁷ The study revealed that, although local immunotherapy significantly reduced conjunctival provocation test scores, it did not alleviate the symptoms and signs of allergic conjunctivitis. Additional studies are thus required to determine the clinical efficacy of local conjunctival immunotherapy.

The introduction of sublingual immunotherapy (SLIT), which is administered at home by the patients themselves without pain and with relative safety, has solved the inconvenience associated with SCIT.^{10,18,19} In general, both SLIT and SCIT are recommended for treatment of seasonal and perennial allergic rhinoconjunctivitis.^{20,21} In a mouse model of allergic conjunctivitis, SLIT with *Dermatophagoides farinae* ameliorated clinical signs, reduced levels of serum antigen-specific immunoglobulin (Ig) E, and interleukin-4 mRNA in the conjunc-



A

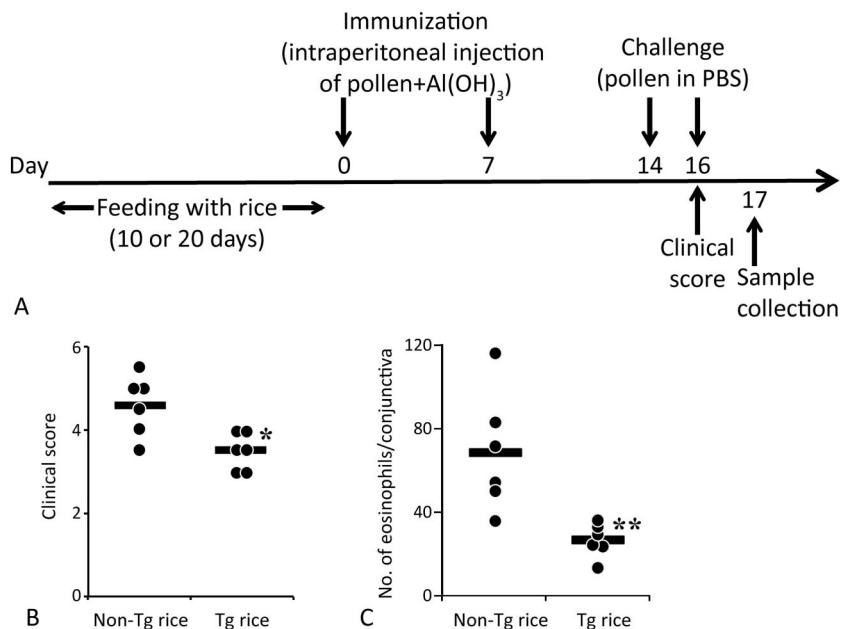


B

FIGURE 2. Loss of B-cell epitopes in modified Cry j 1 and Cry j 2 allergens. A, Dot blot analysis of the indicated amounts of purified native Cry j 1 or Cry j 2, recombinant F1 fragment of Cry j 1 (amino acids 1–144), or molecularly shuffled Cry j 2 with serum from mice immunized with Japanese cedar pollen. Bound IgE was detected with biotin-conjugated antibodies against mouse IgE and streptavidin-conjugated horseradish peroxidase. B, Rat basophil leukemia RBL-2H3 cells were incubated overnight with serum from mice sensitized with Japanese cedar pollen and then stimulated with a total protein extract of Japanese cedar pollen, Cry j 1-F1, or shuffled Cry j 2. The amount of β-hexosaminidase activity released by the cells was measured. From Wakasa et al.³⁸ Reproduced with permission from Wiley.

tiva, and increased levels of serum antigen-specific IgG4 and interferon-γ mRNA in the conjunctiva.²² A double-blind, placebo-controlled study showed that SLIT with a mite extract increased the antigen threshold required for a positive conjunctival provocation test in patients with perennial allergic conjunctivitis.²³ Additionally, a Cochrane systematic review and meta-analysis of many randomized controlled trials, as well as double-masked and placebo-controlled studies performed in Europe and the United States, revealed that SLIT is effective for allergic conjunctivitis.^{24,25} Specifically, it induced significant amelioration of total and individual ocular symptoms (red, itchy, and watery eyes) compared with the placebo.^{24,25} In Japan, SLIT for Japanese cedar pollinosis has only recently been

FIGURE 3. Effects of prophylactic feeding with transgenic rice on the development of experimental allergic conjunctivitis. A, Experimental protocol. Mice were fed daily with 220 mg of transgenic (Tg) rice expressing modified Japanese cedar pollen allergens or with 220 mg of nontransgenic (non-Tg) rice as a control beginning 10 days before intraperitoneal sensitization and subsequent topical challenge with cedar pollen. B, Clinical signs were evaluated by determination of the clinical score at 15 minutes after a second challenge with pollen in phosphate-buffered saline (PBS). C, At 24 hours after the second challenge, eyes were isolated for histological analysis to determine the number of eosinophils in the conjunctiva. Individual circles and bars in (B) and (C) represent mean values for the 2 eyes of each mouse and the overall median values, respectively. * $P < 0.05$, ** $P < 0.01$ (Student *t* test) versus the corresponding value for nontransgenic rice. From Fukuda et al.³⁹ Reproduced with permission from BMJ Publishing Group Ltd.

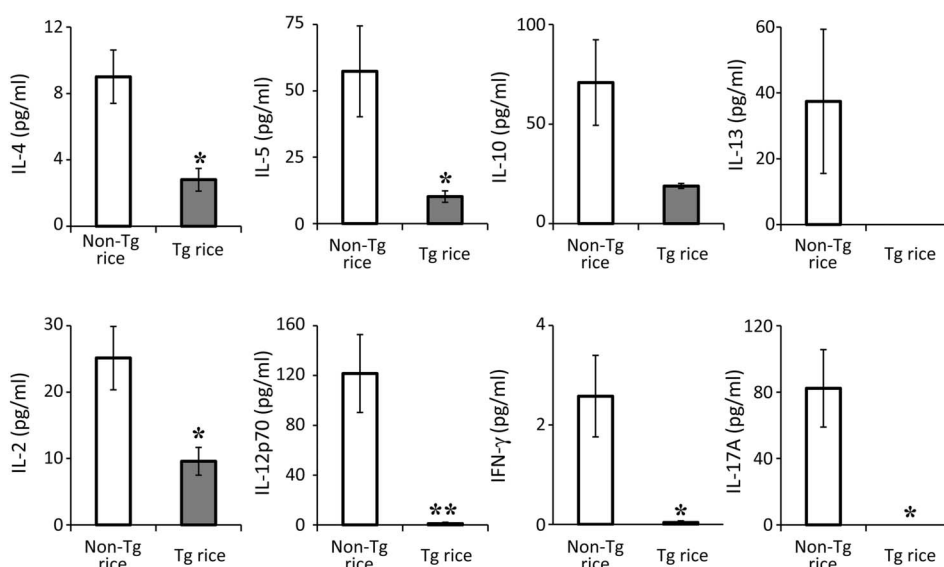


introduced. The first standardized Japanese cedar pollen extract (Cedartolen; Torii Pharmaceutical, Tokyo, Japan) available for SLIT was marketed in 2014. In clinical trials of this extract, SLIT effectively reduced both ocular and nasal symptoms.^{26,27} To date, ~80,000 patients have begun SLIT with this pollen extract. However, the drug is mostly prescribed by otolaryngologists, with few ophthalmologists offering SLIT in Japan. Studies in Europe and the United States have indicated that the compliance rate for SLIT is ~50%,²⁸ whereas a report from Italy revealed that <15% of patients prescribed SLIT actually continue treatment after 3 years.²⁹ Given that such early

discontinuation reduces the efficacy of treatment, this poor compliance is a critical clinical problem.

An ideal immunotherapy for allergic conjunctivitis would be shorter, safer, and more convenient than the currently available approaches. Shortening of the treatment period may require high doses of the antigen, whereas minimization of adverse reactions may necessitate antigen modification. With regard to convenience, and consequent improvements in compliance, oral administration would be preferable to sublingual administration. Therefore, the inclusion of antigens in foods that are consumed every day would be very convenient. We conclude that oral consumption of staple foods

FIGURE 4. Effects of prophylactic vaccination with transgenic rice on systemic cellular immune responses. Mice were fed daily with 220 mg of transgenic (Tg) rice expressing modified Japanese cedar pollen allergens or with 220 mg of nontransgenic (non-Tg) rice as a control beginning 20 days before sensitization and subsequent challenge with cedar pollen as shown in Figure 3A. At 24 hours after the second allergen challenge, the spleen was isolated for measurement of antigen-induced production of cytokines by splenocytes. Data are mean ± SEM for 4 mice per group. * $P < 0.05$, ** $P < 0.01$ (Student *t* test) versus the corresponding value for nontransgenic rice. From Fukuda et al.³⁹ Reproduced with permission from BMJ Publishing Group Ltd. IFN, interferon; IL, interleukin.



engineered to express allergens (edible vaccines) may be an ideal way to deliver antigens for immunotherapy.

ORAL IMMUNOTHERAPY WITH TRANSGENIC RICE AS AN EDIBLE VACCINE

The gastrointestinal tract with its gut-associated lymphoid tissue (GALT) is a frontline of defense against mucosal pathogens that cause infectious disease. GALT also plays a key role in immune tolerance to foods and commensal bacteria.³⁰ Therefore, oral administration of antigens may induce immune tolerance through interaction with GALT. We previously showed that oral antigen administration suppressed antigen-induced allergic conjunctivitis in mice.^{31,32} However, as a result of the action of digestive enzymes in the stomach, the amount of the oral antigen required for suppression was much larger than the amount of the parenterally administered antigen. Vaccines with plant-

based products overcome this disadvantage because the plant cell wall is resistant to digestive enzymes and harsh conditions. Therefore, biopharmaceuticals derived from seeds are suitable as vaccines for oral immunotherapy of allergic diseases.^{33–35}

Given that rice is a staple food in Japan, we have developed rice-based edible vaccines.^{33–37} Expression of antigens in rice seeds for immunotherapy has many advantages. Antigens can be produced at high levels in the edible portion of the seeds when expressed under the control of seed (endosperm)-specific promoters. The antigens are deposited in specialized membrane-bound storage organelles called protein bodies (PBs) and stably accumulate without degradation, even when stored at room temperature for several years. The antigens deposited in PBs are actually protected by 2 barriers, the rigid cell wall and the PB membrane. Given that proteins bioencapsulated in PBs are also resistant to digestive enzymes in the stomach, antigen

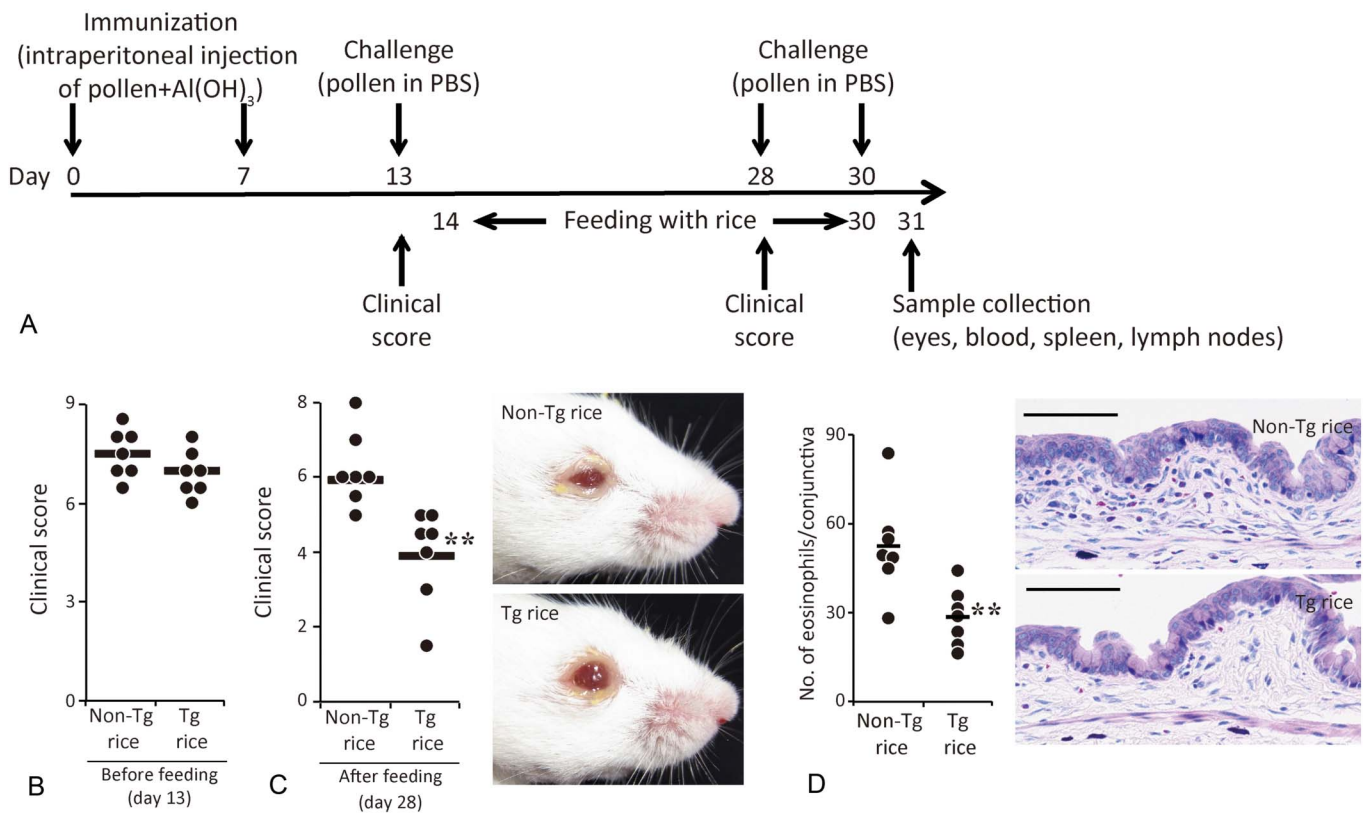


FIGURE 5. Effects of therapeutic feeding with transgenic rice on clinical signs and inflammation in experimental allergic conjunctivitis. A, Experimental protocol. Mice were sensitized twice (days 0 and 7) with Japanese cedar pollen grains mixed with alum, challenged with pollen grains on day 13 and then randomly assigned to 2 groups fed with transgenic (Tg) or nontransgenic (non-Tg) rice before subsequent challenge on days 28 and 30. B, Clinical score evaluated at 15 minutes after the allergen challenge on day 13, before the onset of rice feeding. Circles represent mean values for the 2 eyes of each mouse, and horizontal lines indicate overall median values. C, Clinical score evaluated at 15 minutes after the allergen challenge on day 28, with representative photographs also being shown. $**P < 0.01$ (Student *t* test) versus the value for nontransgenic rice. D, Eyes were subjected to histological analysis with Giemsa staining to determine the number of eosinophils in the conjunctiva on day 31. Representative micrographs for mice fed transgenic or nontransgenic rice are shown. Scale bars, 200 μ m. Individual circles and bars represent mean values for the number of eosinophils in conjunctival sections from the 2 eyes of each mouse and the overall median values, respectively. $**P < 0.01$ (Student *t* test) versus the value for nontransgenic rice. From Fukuda et al.⁴¹ Reproduced with permission from The Japanese Society of Allergology. PBS, phosphate-buffered saline.

peptides in these organelles can be effectively delivered to GALT without getting degraded by these enzymes. Antigens in rice seeds have been shown to retain activity even after autoclave treatment, which means that transgenic rice can be provided to patients as packaged steamed rice that can be heated in a microwave oven before consumption as part of a regular meal every day.

EFFECTS OF TRANSGENIC RICE EXPRESSING HYPOALLERGENIC JAPANESE CEDAR POLLEN ANTIGENS ON ALLERGIC CONJUNCTIVITIS

We recently developed transgenic rice containing entire molecules of the major Japanese cedar pollen allergens, Cry j 1 and Cry j 2, to enable treatment of a broad range of patients with different genetic backgrounds.³⁸ The proteins are expressed as structurally modified forms, with Cry j 1 being fragmented and Cry j 2 being deconstructed by molecular shuffling (Fig. 1). Consequently, their conformational structures are altered in such a way that they retain all T-cell epitopes but lose all B-cell epitopes, which indicates that the modified antigens in the transgenic rice are not recognized by antigen-specific

IgE (Fig. 2A). In addition, the modified antigens do not stimulate degranulation of IgE-binding basophils (Fig. 2B). This reduced risk of anaphylaxis should allow high-dose administration of the modified antigens and therefore shorten the duration of immunotherapy.

We initially examined the potential prophylactic effect of our transgenic rice on Japanese cedar pollen-induced conjunctivitis in mice.³⁹ Mice were fed daily with transgenic rice containing the modified allergens, or with nontransgenic rice as a control, before sensitization with pollen (Fig. 3A). A clinical score based on conjunctival edema and redness, lid swelling, and tearing, all of which are symptoms of human allergic conjunctivitis, was evaluated at 15 minutes after a second antigen challenge as a measure of the early-phase reaction of allergic conjunctivitis.⁴⁰ The clinical score was significantly reduced in mice fed the transgenic rice compared with those fed the nontransgenic rice (Fig. 3B). Eosinophil infiltration into the conjunctiva was assessed as a measure of late-phase inflammation and was also significantly suppressed in mice fed the transgenic rice compared with those fed the nontransgenic rice (Fig. 3C). These results indicate that prophylactic feeding with transgenic rice can attenuate the development of pollen-induced

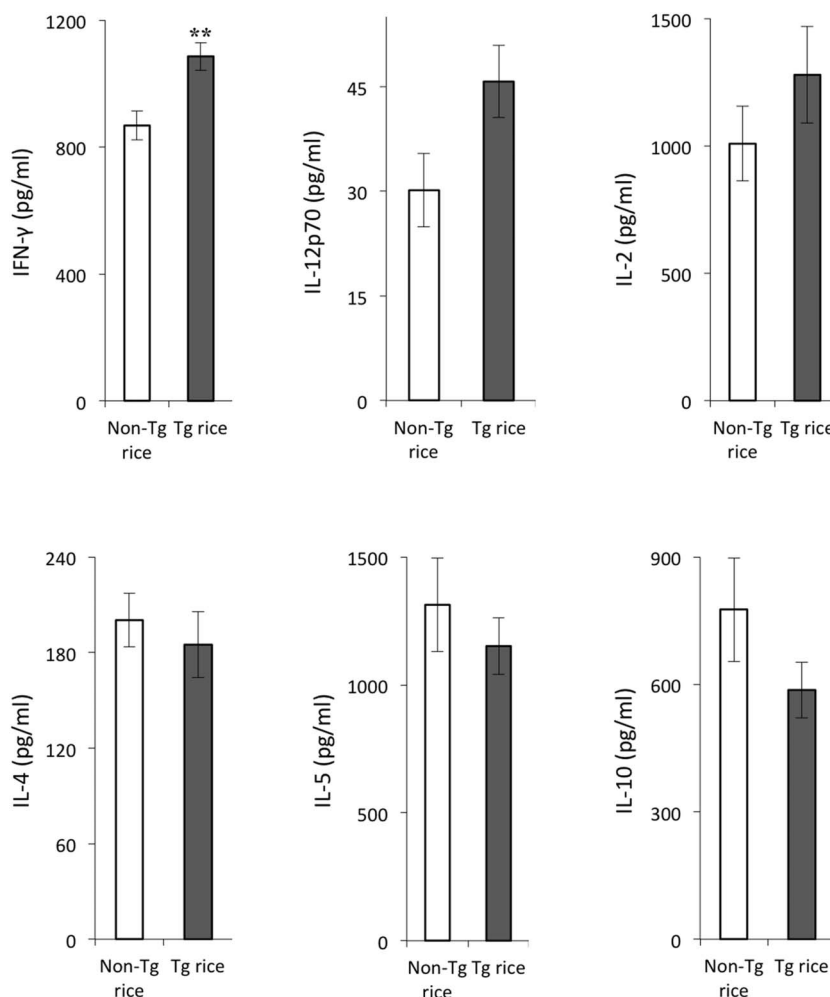


FIGURE 6. Effects of therapeutic feeding with transgenic rice on systemic immune responses. The spleen was isolated at 24 hours after the allergen challenge on day 30 for mice treated as in Figure 5A, and the antigen-induced production of Th1 (IFN- γ , IL-12, and IL-2) and Th2 (IL-4, IL-5, and IL-10) cytokines by splenocytes was measured. Data are mean $\pm$ SEM for 3 mice per group. ** $P < 0.01$ (Student t test) versus the corresponding value for nontransgenic rice. From Fukuda et al.⁴¹ Reproduced with permission from The Japanese Society of Allergology. IFN, interferon; IL, interleukin; Th, T helper.

allergic conjunctivitis. Regarding the systemic immune response, the serum levels of allergen-specific and total IgE were significantly lower in mice fed the transgenic rice. Prophylactic feeding with the transgenic rice also resulted in uniform suppression of antigen-induced production of both T-helper (Th) 2 and Th1 cytokines by splenocytes (Fig. 4). These data suggest that prevention of conjunctivitis in the mice by transgenic rice consumption is likely mediated by the induction of clonal anergy or the deletion of Cry j-reactive T cells rather than by active suppression mediated by regulatory T cells.

We also investigated the effect of prophylactic oral administration of transgenic rice on pollen-induced rhinitis, another key symptom of pollinosis.³⁸ Mice were fed the transgenic or nontransgenic rice daily for 3 weeks before sensitization and intranasal challenge with cedar pollen. The sneezing frequency, as a clinical sign of rhinitis, and numbers of eosinophils and neutrophils in nasal lavage fluid were significantly reduced in mice fed the transgenic rice compared with those fed the nontransgenic rice, indicating that oral administration of transgenic rice can also prevent rhinitis in Japanese cedar pollinosis.

For clinical application of transgenic rice, a therapeutic effect in patients with pollinosis is more important than a preventive effect in healthy individuals. Therefore, we investigated the potential therapeutic effect of oral immunotherapy with transgenic rice on established pollen-induced allergic conjunctivitis in mice.⁴¹ Mice were sensitized and then topically challenged with pollen on day 13 (Fig. 5A). After confirmation of establishment of allergic conjunctivitis, the mice were randomly divided into 2 groups and fed daily with transgenic or nontransgenic rice for 16 days. The mice were then challenged with pollen to evaluate the efficacy of the immunotherapy. Although the clinical score was similar in the 2 groups of mice before rice feeding, it was significantly smaller in mice fed the transgenic rice than in those fed the nontransgenic rice (Figs. 5B, C). Late-phase eosinophil infiltration into the conjunctiva was also significantly suppressed in mice fed the transgenic rice compared with those fed the nontransgenic rice (Fig. 5D). These results showed that therapeutic feeding with transgenic rice can suppress established pollen-induced allergic conjunctivitis. Although prophylactic and therapeutic feeding of transgenic rice similarly ameliorated the clinical signs and conjunctival inflammation, the systemic immune responses differed according to the timing of administration of transgenic rice. The serum concentrations of allergen-specific and total IgE did not differ significantly between the 2 groups of mice subjected to the therapeutic feeding protocol. Furthermore, the numbers of CD4⁺CD25⁺Foxp3⁺ regulatory T cells in the spleen and the submandibular and mesenteric lymph nodes did not differ significantly between the mice fed the transgenic or nontransgenic rice. However, therapeutic feeding with the transgenic rice induced significant upregulation of the antigen-induced production of interferon- γ by splenocytes without affecting the production of Th2 cytokines (Fig. 6). These results suggested that therapeutic feeding with transgenic rice may induce oral tolerance by shifting the Th1–Th2 balance toward Th1-dominant immunity.

CONCLUSIONS AND FUTURE DIRECTIONS

At present, antigen-specific immunotherapy administered by the subcutaneous or sublingual route seems to be effective for suppression of ocular symptoms in allergic conjunctivitis or rhinoconjunctivitis. However, given that both of these approaches have problems—severe side effects and poor compliance, respectively—improvements in therapeutic treatments are desirable. We have demonstrated a novel approach using rice-based allergy vaccines to treat allergic conjunctivitis, in which oral immunotherapy with transgenic rice containing modified Japanese cedar pollen antigens prevented the development of allergic conjunctivitis and suppressed established disease in mice. We have also developed transgenic rice expressing various other antigens, including those of birch pollen, ragweed pollen, and dust mites.³³ Thus, consumption of transgenic rice as part of a regular meal may be an effective, safe, and convenient approach to immunotherapy for allergic conjunctivitis.

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