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REVIEW

Next-Generation Allergen-Specific Immunotherapy for Japanese Cedar Pollinosis Using Molecular Approaches

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Abstract: Japanese cedar (JC) pollinosis is the most major IgE-mediated type I allergic disease in Japan. Allergen-specific immunotherapy is the only curative treatment for allergic diseases. Subcutaneous immunotherapy and sublingual immunotherapy have been introduced in Japan for JC pollinosis, but do not avoid some adverse side effects, because the natural allergens used as tolerogens cross-link with specific IgE types on mast cells and basophils. To make immunotherapy for JC pollinosis safer, more effective and convenient, rice-based oral allergy vaccines using hybrid peptides composed of multiple T-cell epitopes or recombinant deconstructed hypoallergenic derivatives derived from major allergens — Cry j 1 and Cry j 2 — have been developed and their efficacy and safety evaluated by oral administration of transgenic rice seeds. Furthermore, recombinant modified JC allergens conjugated with various immunomodulatory molecules and DNA-based vaccines have been created and their efficacy assessed.

Keywords: allergen-specific immunotherapy, T cell–epitope peptide, Japanese cedar pollinosis, rice-based oral vaccine, hypoallergenic derivatives

Introduction

Japanese cedar (JC; *Cryptomeria japonica*) pollinosis is a predominant seasonal allergic disorder and an important public-health problem in Japan.[1,2] The prevalence of JC pollinosis has increased significantly since the first report in 1964, and affects >30% of Japanese population.[3–5] JC-pollen dispersal usually starts in early February, lasts for 10 weeks, and is followed by Japanese cypress pollen dispersal, which reaches its peak in late March. The major allergens causing JC pollinosis have been identified to be Cry J 1 and Cry J 2. It has been reported that >90% of patients with JC pollinosis have IgE types specific to these allergens. Cry j 1 is a basic glycoprotein homologous to pectate lyase with molecular weights of 41 and 45 kDa and is localized in pollen-cell walls.[6] Cry j 2 is a basic glycoprotein homologous to polymethyl galacturonate with a molecular weight of 45 kDa, which is located in pollen-starch granules.[7,8] There is cross-reactivity between the pollen allergens of JC and Japanese cypress, because about 70% of patients with JC pollinosis are afflicted with Japanese cypress–pollen allergens. This is due to the fact that the Cry j 1 and Cry j 2 allergens share 80% and 74% amino acid–sequence similarities with the homologues of Japanese cypress — Cha o 1 and Cha o 2 — thus suggesting the potential for cross-reactivity for some T-cell and B-cell epitopes.

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Cha o 3 has recently been characterized as a major cypress-pollen allergen in addition to Cha o 1 and Cha o 2.[9] Cry j 4 is a homologue of Cha o 3 (85% identity) with cellulase function and a molecular weight of 61 kDa, but its presence is remarkably low in JC pollen compared to Cha o 3.[10] Several other minor JC-pollen allergens have been also identified.[11] Cry j 3 is a thaumatin-like protein in the PR5 family with a molecular weight of 27 kDa homologous to Jun a 3, a major allergen from mountain cedar pollen. CJP-4 from JC pollen has been identified as a 34 kDa protein with endochitinase activity (class IV chitinase) that cross-reacts with latex allergens. CJP-6 has been identified as an isoflavone reductase–like allergen. The other JC-pollen allergens — CJP-8, CPA9, and CPA63 — have also been identified on allergenome analysis. These are homologous to lipid-transfer protein, subtilisin-like protease and aspartic protease, respectively.

The main treatments for pollinosis are avoidance of pollens and pharmacotherapy using antihistamines, antileukotrienes, and intranasal corticosteroids to block the release of chemical mediators from mast cells and basophils. These treatments relieve symptoms, but do not provide a cure for allergic diseases.

Allergen-specific immunotherapy (AIT) is the only clinically available curative treatment against IgE-mediated type I allergic diseases, such as allergic rhinitis, conjunctivitis and asthma, and exhibits long-lasting effect even after discontinuation,[12] ie, it can change the course of the disease. Immunotolerance to the causative allergens is induced by this treatment, which is conventionally achieved by subcutaneous injection with increasing doses of crude-pollen extract for 3–5 years.[13,14] Used as a tolerogen, this crude extract is also sometimes accompanied by severe side effects, such as anaphylactic shock, which is caused by the cross-linking of allergen with a specific IgE capture of allergen on the surface of mast cells and circulating basophils. Furthermore, frequent medical visits for repeated painful injections are laborious, uncomfortable, and inconvenient. Subcutaneous immunotherapy (SCIT) for JC pollinosis has been administered in Japan since the 1960s.

Sublingual immunotherapy (SLIT) has been proposed as an alternative to SCIT.[15] SLIT reduces the burden on patients because it can be administered at home and has fewer severe side effects. However, it takes longer to attain therapeutic efficiency than SCIT, and its effects are thought to be somewhat weaker.

Therefore, establishment of more convenient, more effective, and safer protocols is highly desired for a next-generation AIT. To overcome these problems, designing recombinant hypoallergenic derivatives retaining immunogenicity, but reducing allergenicity (IgE activity), is an ideal strategy. Destruction of the conformational structure of allergens required by recognition of specific IgE types can be achieved by oligomerization, fragmentation, shuffling (reassembling of sequences), or deletion of disulfide bonds of the causative allergen sequence.[16,17] Hybrid T cell–epitope peptides derived from allergens have been designed.[18] Many clinical trials on allergy patients have been performed using these recombinant hypoallergenic derivatives, and their efficacy and safety have been evaluated in various allergies. Furthermore, conjugation of adjuvants with tolerogens and new routes of administration have been developed to allow improvement of efficacy and convenient administration.

Administration Routes of Allergens for AIT

Subcutaneous Immunotherapy

SCIT typically requires a buildup phase of one to three injections per week for the first 3–6 months of treatment, with maintenance injections every 2–8 weeks afterward for 3–5 years. Nonstandardized JC-pollen extract was launched in 1969 and used in clinical practice until 2000, when its standardization was achieved by the Japanese Allergology Society. SCIT with Japanese Society for Tuberculosis–standardized JC-pollen extracts has led to reduced clinical symptoms and medication scores, as well as improvement in quality of life (QOL). Reduction of JC pollen–specific IgE and basophil activation and elevation of JC pollen–specific IgG_4 levels have been induced by SCIT over the long term (3-5 years).

Sublingual Immunotherapy

SLIT has been accepted as a viable alternative to SCIT for at least 30 years. SLIT can be administered at home, thus providing benefits in convenience and comfort. Theoretically, this aims to avoid the severe acute side reactions induced with large doses. SLIT with a new standardized JC pollen extract for JC pollinosis was approved in October 2014 by the Japanese Ministry of Health, Labour, and Welfare.[21] At present, SLIT for JC pollinosis is achieved by placing the allergen under the tongue via either SLIT drops (Cedartolen) in an aqueous

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formulation of allergen extract or SLIT tablets (CedarCure) in a freeze-dried formulation. JC-pollen SLIT drops (Cedartolen) have usually been used at a dose of 2,000 JAU (Japanese allergy unit) containing 1.5–4.2 µg Cry j 1 as the maximum concentration for the maintenance phase. Although this dose has been reported to be effective in improvement of clinical symptoms for both children and adults, a higher dose is desired to enhance efficacy, because it has been shown to be dose- and duration-dependent.[22,23] To improve clinical efficacy and patient convenience, JC-pollen SLIT tablets 5,000 JAU/day was selected as the optimum dose and is used for treatment during the maintenance phase.[23] However, the immunological effects of SLIT are lower than those of SLIT, since higher doses of the causative allergen (20- to 30-fold) are required for SLIT to obtain the same therapeutic effect as SCIT. SLIT has a better safety profile than SCIT. Actually, the risk of systemic allergic reactions (severe anaphylactic shocks) is extremely decreased, whereas local side effects, such as oral itching and swelling, occur at a relatively high rate.

Large clinical SLIT trials for JC pollinosis have demonstrated significant reductions in total nasal symptoms and medical scores compared to placebo.[24,25] The number of JC pollen–specific T_h2 cytokine-producing cells increased during the pollen season in the placebo group, whereas increases were inhibited in the SLIT group. The number of JC pollen specific–induced $Foxp3^+$ regulatory T cells (T_{reg}) cells increased only in the SLIT group. SLIT induced tolerance to JC-pollen allergens by increasing specific serum IgG_2 and IgG_4 levels and allergen-specific T_{reg} cells producing IL10 and TGFβ.[26] When SLIT is carried out by treatment with JC-pollen SLIT drops for about 3 years, therapeutic effects, such as alleviation of clinical symptoms, reduction in pharmaceutical, use and improvement in QOL are maintained for at least 2 years, even after the cessation of treatment.[27] Such symptomatic improvement is closely associated with the increase in serum allergen–specific IgG_4 acting as blocking antibody to IgE.

Oral Immunotherapy

Oral immunotherapy is a promising method for modulating immunoresponse, since relatively large amounts of allergens can be orally administered and a large number of immune cells are present in gut-associated lymphoid tissue (GALT), providing suitable circumstances to induce immunotolerance to administered allergens.

The oral route is safe and convenient, and usually used as immunotherapy against food allergies. However, oral immunotherapy has been fundamentally abandoned for allergic rhinitis, because satisfactory clinical efficacy at both low and high does could not be obtained for several allergens (birch pollen and timothy-grass pollen allergen) in the 1980s.[28] Side effects affecting the gastrointestinal tract were also reported in many patients in the active-treatment groups. These negative results might have been due to inadequate formulation of the allergens administered and the requirement for high amounts of allergens.

It has recently been recognized that cereal seed provides an effective delivery vehicle for antigens to GALT without severe degradation by digestive enzymes in the gastrointestinal tract when transgenic seed containing antigens (T cell–epitope peptides or hypoallergenic derivatives of JC-pollen allergens) are orally administered.[29] This is caused by the antigen deposited in protein bodies (PBs) of endosperm cells being bioencapsuled through double barriers of the cell wall and PBs exhibiting resistance to proteolytic enzymes and harsh conditions.[30] It is known that recombinant proteins expressed in seed (endosperm) highly and stably accumulate in PBs devoid of proteases when produced as secretory proteins via the endoplasmic reticulum (ER). Furthermore, seed has many advantages as a production platform of recombinant proteins in terms of productivity, stability, safety, cost-effectiveness, and scalability.[31]

The amount or nature of the antigen determines the route of uptake into different parts of GALT such as the lamina propria or Peyer's patches for induction of oral tolerance. Particulate materials enter GALT mostly by M cell–mediated transcytosis, whereas soluble or small antigens are absorbed by epithelial cells and/or taken up by epithelium-associated macrophages, dendritic cells (DCs), or goblet cells. Oral tolerance is initiated by $CD103^+$ DCs that capture the antigen in the lamina propria. After contact with the antigen, $CD103^+$ DCs migrate to the mesenteric lymph nodes and induce differentiation of naïve T cells into gut-forming regulatory Treg cells through a mechanism dependent on TGFβ and retinoic acid.

Regarding the underlying oral immunotolerance mechanism, T_{reg} cells play a critical role in developing and maintaining immunotolerance to allergens by producing inhibitory cytokines (IL10, TGFβ, and IL35).[32,33] Under normal conditions, it is known that repeated exposure at low doses of antigen promotes the development of T_{reg} cells. In contrast, exposure to large amounts of antigen leads to T-cell anergy or Fas-mediated clonal deletion.

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$FOXP3^+$ iT_{reg} cells are essential for establishing oral tolerance on exposure to inhaled allergens, since mutations in FOXP3 locus results in a loss of peripheral tolerance. Other T_{reg} cells, IL10-producing T_r1 cells and TGFβ-producing T_h3 cells are also involved in peripheral oral tolerance. In particular, T_h3 cells are characteristically induced after antigen feeding, and are implicated in the development of $FOXP3^+$ iT_{reg} cells via secreted TGFβ. $FOXP3^+$ iT_{reg} cells can condition DCs to induce IL10-producing T_r1 cells by a mechanism dependent on IL27. T_r1 cells suppress immunoreactions through IL10 production. IL10-producing T_r1 and B_{reg} cells contribute allergen tolerance through suppression of effector T cells (T_h1 and T_h2) and selective induction of blocking IgG_4 antibodies via IL10 production.

Other Immunotherapies Using Noninjection Routes

Intralymphatic immunotherapy (ILIT) is a novel method for direct administration of allergens into lymph nodes. The major benefit of ILIT is the reduced dose of allergen and numbers of injection. Improved safety, efficacy, and convenience of ILIT have been also reported. This immunotherapy has recently been applied to JC-pollinosis patients.[34] JC pollen–allergen extracts were administered by three intralymphatic inguinal injections before the first pollen season. ILIT-treated patients (n=12) tended to show improved symptom and medication scores compared to placebo-treated ones (n=6), though without statistical significance. When assessed by nasal provocation testing and visual analogue–scale scoring after the first season, the effectiveness of ILIT was significant. Notably, these effects continued until the second or third season. Neither allergen-specific antibodies nor T_{reg}/B_{reg} cells changed in peripheral blood.

Epicutaneous immunotherapy is another novel AIT method in which allergens are applied to the skin via patches containing a small dose of allergen. Administered allergens captured by Langerhans cells and epidermal DCs migrate to lymph nodes to prime $CD4^+$ T cells. To date, this administration route has not yet been applied to AIT for JC pollinosis. This treatment has been put forward for immunotolerance induction against food allergies, such as peanut allergy.[35]

Modification of Allergens (Design of Allergy Vaccines)

T Cell–Epitope Peptides

T-cell epitopes were originally designed to modulate allergen-specific T-cell responses without IgE-mediated activation of effector cells. This was due to evidence that T cell–epitope peptides retained immunogenicity, but had little ability to cross-link the allergen-specific IgE (IgE reactivity) on mast cells and basophils. As a result, T-cell epitopes can induce T-cell tolerance without side effects.

The underlying immunological mechanism of T cell epitope–based peptide immunotherapy is induction of specific anergy or deletion of allergen-specific T_h2 cells, immunodeviation, and induction of T_{reg} cells, leading to IL10 production.[36] Allergen-specific protective IgG_4 acting as a blocking antibody against IgE is not elicited, due to the short length of the peptide. Such an immunotolerance mechanism is associated with administration duration, tolerogen dose, and administration route. Repeated administration at low doses induces T_{reg} cells, which is called active suppression, while high-dose administration leads to T-cell anergy and deletion of effector T cells. T_{reg} cells are involved in inducing and maintaining specific unresponsiveness to allergens. Unresponsiveness is primarily maintained by IL10 and TGFβ, which are produced by antigen-specific T_{reg} cells. Naturally occurring $FOXP3^+$ and induced Treg cells (iTreg cells) contribute to the establishment of peripheral T-cell tolerance.

As for the efficacy of peptide oral immunotherapy for JC pollinosis, it was first reported that oral administration of Cry j 2 major T cell epitopes (p246–259) to model BALB/c mice before challenge with the Cry j 2 allergen resulted in reduction of T-cell proliferation and specific IgE and histamine release by mast cells.[37] Alleviation of sneezing and airway obstruction were also observed.[38] These results indicated that oral feeding of T cell–epitope peptides can induce immunotolerance in specific T cells. However, to obtain a similar effect to SCIT, >100-fold doses of tolerogen were required

To test a new immunotherapy based on the principle of seed-based oral tolerance, it was examined whether rice seed containing major T-cell epitopes derived from Cry j 1 and Cry j 2 allergens could be utilized as a vehicle to deliver the tolerogen to GALT through oral administration. Major mouse T-cell epitopes from two allergens (p277-290 of Cry j 1 and p246-259 of Cry j 2) were expressed as part of the soybean storage protein glycinin ($A_{1a}B_{1b}$) under the control of the endosperm-specific rice glutelin *GluB1* promoter in transgenic rice seed.[39] When transgenic rice seeds were fed to BALB/c mice for 4 weeks prior to systemic challenges with JC pollen, not only significant reductions in specific T cell–proliferative reactions but also

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suppression of allergen-specific IgE and IgG levels and production of T_h2 cytokines, IL4, IL5, and IL13 were induced compared to those of mice fed with nontransgenic rice seeds. Release of histamine from mast cells and sneezing regularity were restricted by oral administration of transgenic rice seeds.[39] These results indicated that oral immunotolerance was definitely induced by oral administration of transgenic rice seeds containing T-cell epitopes.

For application of peptide immunotherapy to patients with JC pollinosis, multiple major T-cell epitopes were selected to overcome the variation of T-cell epitopes on the basis of different human leukemia–antigen (HLA) class II restrictions. Artificial hybrid peptides consisting of five to seven major human T cell epitopes with length of 13–25 amino acids derived from Cry j 1 and Cry j 2 were synthesized by recombinant technology.[40–42] These hybrid peptides were designed to prevent IgE-mediated adverse effects. When the safety and efficacy of these hybrid peptides were evaluated, it was found that they did not bind to specific IgE antibodies in sera from patients with JC pollinosis, indicating no allergenicity of these hybrid peptides.-[40] Furthermore, they had T cell–proliferative activity comparable to the native Cry j 1 and Cry j 2 allergens. It is notable that the hybrid peptide had higher T cell–proliferation activity than the mixture of T-cell epitopes.[40]

The therapeutic efficacy of a hybrid peptide composed of six major human T-cell epitopes from Cry j 1 and Cry j 2 (Cry-consensus peptide) was examined by subcutaneous administration once a week for 5 weeks to B10.S model mice sensitized with Cry j 1 and alum.[42] In sensitized B10.S mice, a major T cell–epitope (peptides (p)211–225) of Cry j 1 coincided with one of the prevalent epitopes of the Cry-consensus peptide. The hybrid peptide inhibited the production of Cry j 1-specific IgE, IL4, and IL5 (T_h2-mediated) and enhanced Cry j 1-specific IgG_{2a} and IFNγ levels (shift to T_h1 immunoresponse). Cry j 1-induced sneezing and eosinophil infiltration in nasal tissues were also depressed. These results indicated that this hybrid peptide can induce immunotolerance to specific T cells.

Then, a hybrid peptide consisting of seven major human T-cell epitopes (7Crp) from Cry j 1 and Cry j 2 was produced in transgenic rice seeds and used as an oral vaccine.[43] To enhance accumulation in the edible part (endosperm) of rice seed, the codon-optimized 7Crp synthetic gene was expressed as a secretory protein by ligating the signal peptide and KDEL ER retention signal at its N and C termini under the control of the glutelin endosperm–specific promoter.[43] The 7Crp peptide was mainly deposited in ER-derived PBs in rice endosperm, whereas it did not accumulate in other tissue, such as leaf and stem, when expressed under the control of the ubiquitin constitutive promoter.[44]

The efficacy of transgenic rice containing 7Crp was evaluated by oral administration to model mice and then subjected to clinical study in human subjects. When transgenic seeds were orally administered to B10.S mice with one common major T-cell epitope (p211–225 of Cry j 1), and then nasally challenged with Cry j 1 allergen, Cry j 1 specific T cell–proliferative response and specific-IgE levels were decreased compared with control mice fed nontransgenic rice seeds.[43] This suggests that the T cell-epitope in the hybrid peptide has the potential to suppress allergen-specific immunoreaction through the induction of immunotolerance to specific T cells (linked epitope suppression).

When the first clinical study of this transgenic rice in JC-pollinosis patients had been completed, it was found that allergen-specific T-cell proliferations against Cry j 1 and Cry j 2 allergens were significantly downregulated within just 2 weeks.[45] This suppressive effect was dose-dependent. On the other hand, when 80 g transgenic rice was orally administered for 20 weeks from December to the end of March, IL10 upregulation and IL13 downregulation were not associated with decreased cedar pollen–specific IgE antibodies or increased IgG_4-blocking antibodies. There was no effect on IFNγ and IL5 secretions from T_h1 and T_h2 cells, and the effect on IL4 was also minimal. This treatment did not accomplish improvement medication and QOL symptom scores. Significant improvements in symptom scores was restricted to the nose and eyes itching. As such, even though T cell–proliferative activity to Cry j 1 and Cry j 2 were highly suppressed, 80 g transgenic rice for 20 weeks did not give rise to a profound clinical improvement.

To examine the clinical efficacy of low-dose intake of transgenic rice for longer duration, two doses of transgenic rice seeds (5 g or 20 g) were orally administered to patients for 24 weeks in each of two successive JC-pollen seasons.[46] Specific T-cell proliferation following stimulation with 7Crp, Cry j 1, and Cry j 2 was significantly suppressed in the second JC-pollen season. No significant differences were detected among the three groups (5 g, 20 g, and placebo) in the first season on clinical symptoms or medication scores. However, medication and face-scale scores improved in the 5 g transgenic rice group in the second season.

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These partial immunotolerance effects may be due to the possibility that 7Crp does not cover the full repertoire of T-cell epitopes localized in Cry j 1 and Cry j 2 molecules due to the diversity of MHC (HLA) class II alleles in patients. Therefore, inclusion of additional T-cell epitopes to address MHC diversity may be necessary to accomplish significant clinical improvement. At present, transgenic rice expressing hybrid peptides composed of 12–13 T-cell epitopes covering most human T-cell epitopes has been generated.[47] This transgenic rice may be expected to be broadly applicable as a next-generation oral peptide vaccine.

Recombinant Hypoallergenic Derivatives

Many B-cell epitopes are conformational, such that IgE reactivity is dependent on their correctly folded tertiary structure. As such, to reduce the allergenicity of Cry j 1 and Cry j 2, their tertiary structures have been deconstructed by fragmentation, shuffling (reassembly), and mutagenesis of Cys residues implicated in disulfide bonds. Transgenic rice expressing all molecules of deconstructed Cry j 1 and Cry j 2 was generated to treat a broader range of patients with different genetic backgrounds.[48,49] These modified allergens preserve most T-cell epitopes, but lack B-cell epitopes. The reduction of allergenic and inflammatory activity from these allergens has been evaluated by IgE-binding assays, basophil-degradation tests, and basophil-activation tests.[48–50]

Tolerogenic efficacy of transgenic rice seed expressing the deconstructed Cry j 1 and Cry j 2 allergens has been evaluated in a murine model of allergic rhinitis.[48] When mice fed transgenic seeds daily for 3 weeks were then challenged with JC pollen, allergen-specific $CD4^+$ T-cell proliferation and specific IgE and IgG levels were significantly suppressed compared with mice fed nontransgenic rice seeds. It is notable that induction of blocking IgG_4 or IL10 secreted from T_{reg} cells was not detected, although production levels of the T_h2 cytokines IL4, IL5, and IL13 were significantly depressed. Clinical symptoms of pollinosis, sneezing frequency, and infiltration of inflammatory cells (eosinophils and neutrophils) in nasal tissue were alleviated compared to controls. These results indicated that oral administration of transgenic rice seeds can induce immunotolerance to JC-pollen allergens, thus providing a promising approach using AIT for JC pollinosis.

Such prophylactic effects after transgenic rice intake have been also ascertained in an allergic conjunctivitis murine model.[51] When mice were fed once a day for 20 days with transgenic rice seeds and then sensitized with two intraperitoneal injections of JC pollen, numbers of eosinophils and total inflammatory cells in the conjunctiva were significantly reduced in mice fed the transgenic rice seeds. Serum concentrations of both total and allergen-specific IgE were also significantly suppressed in mice fed the transgenic rice seeds. Furthermore, oral vaccination with transgenic rice resulted in significant downregulation of allergen-induced production of IL2, IL4, IL5, IL12p70, IFNγ, and IL17A by splenocytes, indicating the suppression of effector T_h1, T_h2, and T_h17 cells. These results indicate that pollen-induced experimental allergic conjunctivitis in mice can be suppressed by oral administration of transgenic rice seeds.

Not only prophylactic but also curative effects have been observed after oral administration of this transgenic rice. When model mice were sensitized with JC pollen and then fed for 16 days with transgenic rice seeds, the number of eosinophils in the conjunctiva and clinical scores for conjunctivitis were significantly reduced compared to mice fed nontransgenic rice seeds.[52] Although T_h2-cytokine IL4 and IL5 levels were not affected, IFNγ was significantly enhanced. On the other hand, there was no significant difference in the number of $CD4^+CD25^+FOXP3^+$ T_{reg} cells in the spleen or lymph nodes between mice fed transgenic and non-transgenic rice. These results suggested that increased IFNγ production might contribute to suppression of conjunctival eosinophil infiltration in mice fed the transgenic rice.

Oral administration of transgenic rice containing deconstructed Cry j 1 and Cry j 2 demonstrated that blocking IgG_4 or IL10 was not induced. This is different from the immunotolerance mechanism via IgG_4 and T_{reg} cells observed in SCIT or SLIT using crude-pollen extract. Results from OIT using rice seeds suggested that immunotolerance may be induced through anergy or deletion of antigen-specific T cells. This difference may to be related to the antigen dose, antigen formulation, administration frequency, or administration route.

Next, before clinical studies in humans, transgenic rice seeds were orally administered to naturally sensitized Japanese monkeys exhibiting similar clinical allergic symptoms.[53] Curative efficiency was suggested from results showing that suppression of allergen-specific PMBC proliferation was observed within just 2 months and allergen-specific IgE levels decreased in some monkeys. Furthermore, oral administration of transgenic rice to Japanese monkeys with JC pollinosis or healthy monkeys did not induce side effects. These findings indicate that

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transgenic rice containing hypoallergenic Cry j 1 and Cry j 2 serves as a safe tolerogen.

DNA Vaccines for AIT

Nucleic acid–based vaccines have been shown to induce an immunological bias that prevents T_h2 sensitization and drive immunoresponse toward T_h1 via induction of IFNγ and IgG_{2a} and suppression of IgG_1, IgE, and allergic inflammation. Furthermore, it is expected that nucleic acid–based vaccines have less risk of inducing severe side effects, such as anaphylaxis.

Repeated intramuscular inoculation of BALB/c mice with pCACJ1 (plasmid DNA encoding Cry j 1) elicited a predominant T_h1-type immunoresponse (IgG_{2a} production) and suppressed IgE and IgG_1 responses.[54] Splenic T cells isolated from pCACJ1-inoculated mice secreted IFNγ but not IL4 upon stimulation with Cry j 1, as well as its major T-cell epitope (p277–288). These findings suggest that intramuscular inoculation with pCACJ1 effectively elicits Cry j 1-specific T_h1-type immunoresponses, resulting in inhibition of the IgE response to Cry j 1.

To establish a new immunotherapy for type I allergic diseases without allergic side effects, MHC class II targeting DNA vaccine was developed using an invariant chain (Ii). This DNA vaccine for JC pollinosis was composed of the Cry j 2 major T-cell epitope and Ii for the delivery of the epitope peptide into the MHC class II–loading pathway. The Cry j 2 major T-cell epitope (p247–258) was inserted into the Ii by replacement with the core of class II-associated invariant chain peptide (CLIP) or by fusion to the C terminus of the Ii, called pCPCJ2 and pliCJ2, respectively.[55] When BALB/c mice were repeatedly inoculated with these plasmid DNAs intramuscularly, a T_h1-cell response specific to p247–258 was induced without eliciting IgG-antibody production, resulting in inhibition of the subsequent IgE response to Cry j 2 immunization.

Lysosomal-associated membrane protein-1 (LAMP-1) is known to be a resident protein of lysosomes. Its lysosome-targeting property has been used in the DNA-vaccine field. It has been shown that inclusion of LAMP significantly enhances both cellular and humoral responses in vaccinated animals. When DNA vaccines encoding CryJ1–LAMP and CryJ2–LAMP fusions were administered to BALB/c mice, high levels of IFNγ and anti-Cry j 1/anti-Cry j 2–specific IgG_{2a} and low levels of IgE were induced, suggesting that T_h1 response was predominantly elicited.[56] After repeated allergen exposure, vaccinated mice were well protected, as indicated by a minimal level of allergen-specific IgE. These results suggest that the CryJ–LAMP vaccine has potential as effective therapy for JC pollinosis by skewing the allergy reaction from T_h2 toward a T_h1-dominant response. Robust and long-lasting T_h1-type immunoresponse is expected to be triggered by LAMP-based DNA vaccination.

Safety and immunological effects of the CryJ2–LAMP DNA vaccine were evaluated in phase IA and IB clinical trials.[57] Both JC and/or mountain cedar atopic human subjects were vaccinated with CryJ2-LAMP plasmid 4 times in the Phase IA trial and some subjects were boosted once in the Phase IB stage. The safety and immunologic biomarkers were assessed by adverse events, skin prick test for allergens and antibody detection in these subjects for an accumulated time from Day 0 of the Phase IA to the end of the Phase IB. The results indicated that CryJ2-LAMP DNA vaccine is safe and may be immunologically effective in treating JC-induced allergy.

Allergens Coupled with Immunomodulatory Compounds

Galactomannan-Coupled Allergens

When orally administered, conjugation of Cry j 1 with galactomannan has been shown to protect against Cry j 1 protein enzymatically digesting in the stomach. No severe adverse events have been observed in subjects receiving Cry j 1–galactomannan.[58] This is due to the fact that IgE-recognition sites in Cry j 1 are masked by the conjugated galactomannan. Furthermore, galactomannan is known to accelerate the uptake of Cry j 1 antigen into gut DCs. When orally administered to patients with JC pollinosis for 4 months from January to March, allergen-specific IgG_4 and IL10 production were enhanced in PBMCs when compared to the control group. Furthermore, improvement in QOL scores and reductions in total symptom scores, medication scores, and total symptom-medication scores were observed throughout the pollen season.[59] These results indicated that oral administration of Cry j 1–galactomannan conjugates provides rapid, effective, and convenient immunotherapy. Galactomannan acts as a carrier and adjuvant for induction of tolerance to JC-pollen allergen.

CpG-Conjugated Allergens

Conjugating immunostimulatory sequences, such as CpG motifs,with allergens has been reported to enhance the effect of DNA for AIT. These sequences bind to toll like receptor 9 (TLR9), which is expressed within endosomes

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of human plasmacytoid DCs and B cells and thus promotes T_h1 responses. Therefore, the allergenic activities of allergens are expected to be reduced by coupling of CpG-motif sequences with allergens.

When Cry j 1 was conjugated with CpG oligodeoxynucleotide and tested in a mouse model, strong allergen-specific T_h1 responses, such as Cry j 1-specific IgG_{2a} response and production of IL12, were elicited in their splenocytes.[60] Moreover, higher IFNγ levels were produced by $CD4^+$ T cells of spleens in mice stimulated with CpG conjugated with Cry j 1. These results indicated that Cry j 1–CpG immunization can induce Cry j 1-specific T_h1 immunoresponses. Furthermore, CpG conjugation reduced binding to IgE in JC-pollinosis patients, indicating IgE epitopes on Cry j 1 were masked by CpG. As a result, Cry j 1-specific IgE levels were suppressed by CpG–Cry j 1 treatment.

Furthermore, T-cell epitopes derived from Cry j 2 was conjugated with CpG oligodeoxynucleotide and tested in mice. T-cell epitope–CpG treatment suppressed Cry j 2-specific IgE reactions and attenuated clinical symptoms upon allergen challenge.[61] T_h2-cytokine response (IL4 and IL5 production) was also significantly suppressed.

Oligomannose-Coated Liposomes Entrapped with Allergens

Antiallergenic effects following treatment with Cry j 1 entrapped with oligomannose-coated liposomes (Cry j 1–OMLs) were investigated in BALB/c mice sensitized with Cry j 1.[62] When unsensitized mice were pretreated with Cry j 1–OMLs and then sensitized with Cry j 1, total and allergen-specific IgE levels were suppressed. Injection of Cry j 1-OMLs induced robust T_h1-cytokine responses (IFNγ production) and Cry j 1-specific IgG_{2a} (T_h1-type antibody) production. These results indicated that allergen-specific T_h1 immuneoresponses were preferentially induced with OMLs.

Polyethylene Glycol–Conjugated Allergens

To destroy the Cry j 1 and Cry j 2 conformational structures, the allergens were fused. Furthermore, a majority of Cys residues were replaced with serine residue to delete disulfide bonds required for maintenance of tertiary structures.[63] This modified Cry j 1–Cry j 2 fusion protein was synthesized in *Escherichia coli* and conjugated with polyethylene glycol (PEG). When the purified PEGylated Cry j 1–Cry j 2 was subcutaneously injected into Cry j 1-sensitized mice and JC pollen–sensitized monkeys and then subcutaneously or intranasally challenged with Cry j 1 or JC pollen, Cry j 1-specific IgE levels were significantly suppressed, while CryJ1-specific IgG levels were increased in the PEGfusion–treated group. When splenocytes were stimulated with Cry j 1, Cry j 1-specific T-cell proliferation and T_h2 cytokine (IL5, IL13) production were significantly reduced, whereas T_h1-type IFNγ was significantly increased. These results indicated PEG–Cry j 1–Cry j 2 elicited a T_h1-skewed immunoresponse. Therefore, PEG fusion is a suitable candidate as a safer and more effective vaccine for AIT.

CTB-Fused Allergens

Cholera toxin B (CTB) subunit has been used as an efficient mucosal carrier molecule for induction of oral tolerance. A mouse-type hybrid T cell–epitope peptide (3Crp) from Cry j 1 and Cry j 2 was expressed in rice seed as a fusion protein with either CTB or rice glutelin as a control. When these transgenic rice seeds were orally administered to BALB/c mice and then challenged with JC pollen, rice seed containing CTB/3Crp suppressed allergen-specific IgE responses and pollen-induced clinical symptoms (histamine release, sneezing frequency) at 50-fold lower doses than required when using control seed.[64] These results indicated that the linked CTB had adjuvant activity for induction of oral immunotolerance.

Conclusion

AIT is the treatment of allergic disease by suppressing the immune system. The basic principle of AIT is to induce immunotolerance (a state of unresponsiveness of the immune system) to allergens through multiple cellular and molecular mechanisms by administering causative allergens. Immunotolerance is known to be regulated by such factors as T-cell anergy, apoptosis, T_{reg} cells, B_{reg} cells, suppressive cytokines, and DCs. SCIT and SLIT are used as effective treatment of various pollen-allergy diseases, including JC pollinosis, which leads to long-lasting effects even after cessation of treatment. SLIT has come to be the main one used because it is safer, painless, and comfortortable. However, there remain several drawbacks, such as risk of adverse reactions, long treatment duration (>3 years), and low efficiency.

To overcome these problems observed in SCIT and SLIT, it is necessary to develop allergy vaccines with high efficiency (immunogenicity) without side effects

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(IgE reactivity). Furthermore, to improve immunoefficacy and patient convenience, administration routes and adjuvants leading to immunoenhancement also have to be taken into consideration (Figure 1).

Regarding efficacy and safety, allergenicity in IgE-binding sites (B-cell epitopes) causing adverse reactions have been eliminated or reduced by destroying the tertiary structure of allergens while retaining the immunogenicity. Reduced allergenicity of destroyed Cry j 1 and Cry j 2 can be confirmed in vitro by IgE-binding and basophil-activation assays. Utilization of recombinant hypoallergenic derivatives or hybrid T cell–epitope peptides composed of multiple major T-cell epitopes derived from causative allergens can be expected to enhance efficacy and safety compared to native allergens. This can be explained by the fact that high-dose administration of allergen with reduced allergenicity (hypoallergenic derivative) allows reduced treatment duration, leading to patient convenience. However, late-phase side effects are still observed for immunotherapy using such hypoallergenic derivatives. This is due to late-phase allergic symptoms caused by T-cell activation based on preservation of T-cell epitopes.

To eliminate both IgE- and T cell–mediated side effects, a carrier-bound B cell epitope–based allergy vaccine has been developed and a clinical trial is already under way grass-pollen allergy.[65] This vaccine is produced by fusing hypoallergenic allergen peptides (B-cell epitopes of length 20–40 amino acids) derived from IgE-binding sites with an immunogenic viral carrier protein. This vaccine reduces IgE- and T cell–mediated side effects and induces robust allergen-specific IgG_4-blocking antibodies. Such a B cell epitope–based allergy vaccine for JC pollinosis has not yet been developed. Conjugation of adjuvant with tolerogens improves efficacy and safety through a shift to T_h1 or modulation of immunological properties, leading to reduction in administered doses and treatment duration. The administration route of tolerogen is associated with not only uptake by key immunoeffector cells but also patient convenience. Therefore, given that

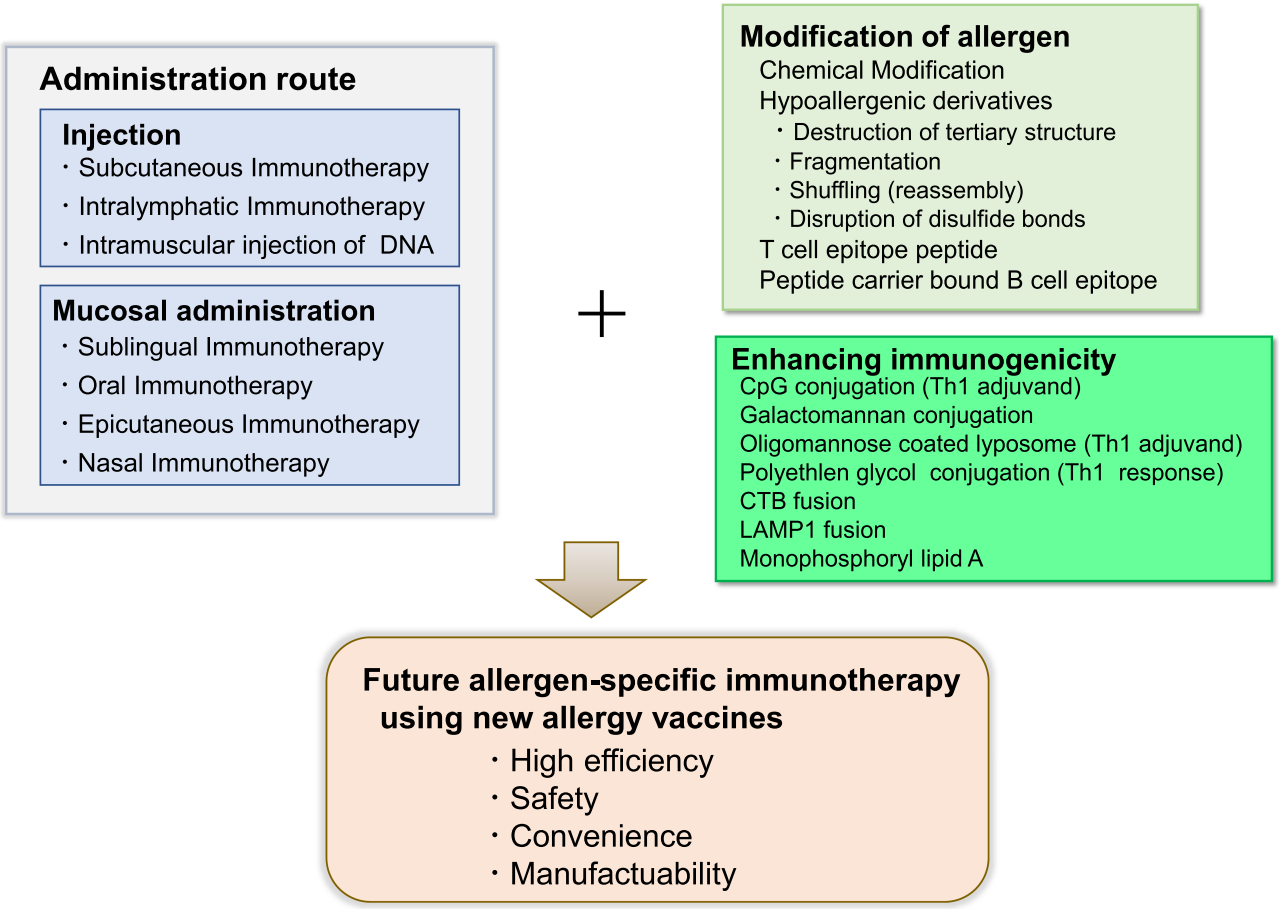

Figure 1 Strategy for development of next-generation allergy vaccines. Next-generation allergen-specific immunotherapy for typeI allergies, including JC pollinosis, that exhibits high efficiency, improved safety, and patient convenience, has to be developed through modification of allergens and use of suitable adjuvants. The administration route also has to be taken into account.

hypoallergenic derivatives conjugated with suitable adjuvant molecule are administered using an appropriate route, immunotolerance to allergens can be effectively induced by much lower doses for shorter durations, resulting in safe, comfortable, and effective AIT for JC pollinosis.

Disclosure

The author reports no conflicts of interest in this work.

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