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Invited review article

Spectrum of allergens for Japanese cedar pollinosis and impact of component-resolved diagnosis on allergen-specific immunotherapy

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ABSTRACT

The high prevalence of Japanese cedar pollinosis in Japan is associated with a negative impact on the quality of life of patients, as well as significant loss of productivity among the workforce in early spring, thus representing a serious social problem. Furthermore, the prevalence is increasing, and has risen by more than 10% in this decade. Cry j 1 and Cry j 2 were identified as the major allergens in Japanese cedar pollen (JCP), and in 2004, the existence of other major and minor allergens were revealed by a combination of two-dimensional electrophoresis and immunoblotting analysis. Allergenome analysis identified a chitinase, a lipid transfer protein, a serine protease, and an aspartic protease as novel IgE-reactive allergens in patients with JCP allergy. Thaumatin-like protein (Cry j 3) was shown to be homologous to Jun a 3, a major allergen from mountain cedar pollen. Isoflavone reductase-like protein was also characterized in a study of a JCP cDNA library. The characterization of component allergens is required to clarify the sensitizer or cross-reactive elicitor allergens for component-resolved diagnosis (CRD). Increasing evidence from numerous clinical trials indicates that CRD can be used to design effective allergen-specific immunotherapy. In this review, we summarize the eight characterized JCP allergens and discuss the impact of CRD and characterization of novel allergens on allergen-specific immunotherapy.
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Prevalence of Japanese cedar pollinosis in Japan

Japanese cedar (*Cryptomeria japonica*) pollinosis is one of the most prevalent forms of seasonal rhinitis in Japan. Japanese cedar pollen (JCP) is released from the male flowers of Japanese cedar trees and levels are usually high from February to April in Japan. During this period, the forecasted levels of JCP in each Japanese geographic prefecture are broadcast with weather reports on a daily basis, and many people choose to wear face masks when they venture outside.[1] Despite the high prevalence of pollinosis and huge interest in the conditions of JCP dispersal in recent decades, pollinosis was first reported in Japan in 1961 as an allergy to ragweed pollen.[2] Subsequently, Japanese cedar pollinosis was discovered in the Nikko area of Tochigi prefecture in 1964.[3] In the past half-century, JCP levels and the prevalence of pollinosis have increased dramatically.[4] The results of a nationwide survey in 2001 using cross-sectional random sampling methods showed that the estimated prevalence of Japanese cedar pollinosis was 13.1%.[5] The most recent survey conducted in 2008 revealed that the prevalence of pollinosis in the Japanese population had almost doubled to 26.5%.[6] Pollinosis has a negative impact on quality of life[7]; therefore, the recent increase in the prevalence of Japanese cedar pollinosis represents a significant social problem in Japan.

Japanese cedar and cypress are major constituents of Taxodiaceae family in Japan. Mountain red cedar, European cypress, and Rocky mountain junipers in Mediterranean countries and United States are also members of Taxodiaceae and Cupressaceae family.[8–11] Patients allergic to pollen from a member of Taxodiaceae/Cupressaceae family shows allergic symptoms after

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inhaling pollen form another member of the family. Group 1, 2, and 3 allergens from pollen in the family cross-react among the pollinosis patients.

Sensitization with pollen allergens

Pollinosis is triggered by the invasion of the nasal and ocular mucosa by pollen grains. Many allergens with the capacity to bind immunoglobulin E (IgE) have been identified from many plant species and are registered on allergen databases.[12] Some pollen-derived allergens are species-specific, while others are commonly identified in various plant species as enzymes or components of grasses, trees, vegetables, and fruits; these are known as pan-allergens. Pollen grains readily access the aqueous phase of the nasal and ocular mucosal membranes, where they are hydrated. The hydrated pollen grain ruptures, releasing their cytoplasmic components, including allergens, non-allergenic proteins, starch granules, and certain chemicals.[13] Non-proteinous components, such as pollen cytoplasmic granules and pollen-associated lipid mediators, may act as adjuvants in the induction of antigen-specific type II helper T cell (Th2)-skewing immune responses to cytoplasmic and surface proteins of pollen during the sensitization phase.[14–16] Some proteins also act as adjuvants in the induction of Th2 responses, accumulation of inflammatory cells, and activation of innate immune cells. Pollen contains proteases in its cytoplasm; this class of enzymes has recently been reported to be important adjuvants of innate and adaptive immune responses via alarm cytokine (alarmin) signals. Serine and cysteine proteases, including papain, stimulate epithelial cells at the mucosal surface and induce the release of thymic stromal lymphopoietin (TSLP) via protease-activated receptor 2 (PAR2) activation. TSLP is expressed mainly by endothelial cells and keratinocytes, and its expression is promoted by IgE, Th2 cytokines including interleukin (IL)-4 and IL-13, and alarmins including IL-25 and IL-33.[17] TSLP can activate myeloid-derived dendritic cells (mDC), which then prime CD4$^+$ T cells to differentiate into antigen-specific Th2 cells in a process orchestrated by OX40-OX40L interactions. Furthermore, TSLP can directly activate naïve CD4$^+$ T cells to promote proliferation and differentiation to the Th2 phenotype through induction of IL-4 following T cell receptor (TCR) stimulation.[18] Protease-stimulated epithelial cells also secrete alarmins with or without the induction of necrosis.[19,20] IL-25, IL-33, and TSLP can activate innate immune cells in a process orchestrated by other cytokines in different manners; IL-25 alone induces inflammatory group 2 innate lymphoid cells (iILC2), while natural helper (NH) cells are strongly activated by a combination of IL-25 and IL-2. A combination of IL-25 and IL-33 induces and activates group 2 innate lymphoid cells (ILC2), while IL-33 alone increases NH cell numbers.[21] Activated ILC2 secrete large amounts of IL-5 and IL-13, which promote the differentiation and activation of naïve CD4$^+$ T cells into Th2 cells and inflammatory effector cells.[22] These reports strongly suggest that pollen contains proallergic natural adjuvants provoking type 2 immunity during the sensitization and elicitation phases of pollinosis (Fig. 1).

In addition to mucosal sensitization, the transcutaneous route may also be important for sensitization in dermatitis and food allergy. In Japan, people who use facial soap containing hydrated wheat protein on a daily basis can develop wheat-dependent exercise-induced anaphylaxis (WDEAI) after the ingestion of wheat-containing food. These patients react mainly to γ-gliadin and ω1.2-gliadin from hydrated wheat protein contained in the product, while patients diagnosed with conventional wheat protein-dependent WDE without using the product react strongly to ω5-gliadin. The patients with hydrated wheat protein-WDEAI sensitized by the soup showed more severe systemic allergic reactions

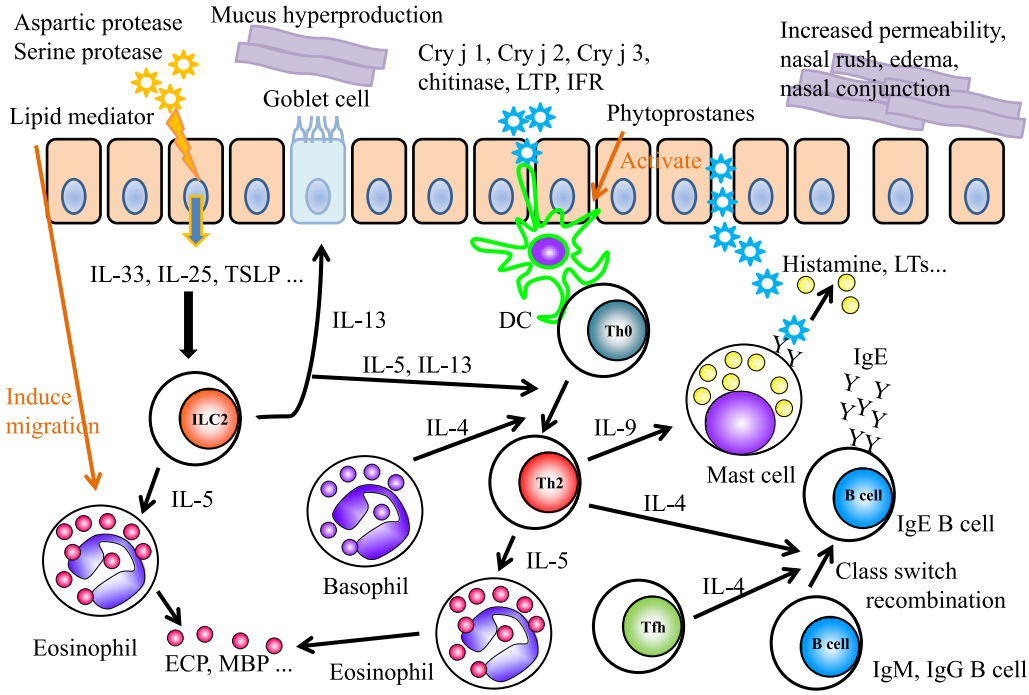

Fig. 1. Schematic hypothetical model of sensitization with Japanese cedar pollen. Pollen protease-mediated damage to the epithelial cells of the nasal mucosa induces the production of cytokines such as IL-33, which in turn induces the migration and activation of inflammatory cells and innate lymphoid cells. Th2 cytokines from ILC2 and inflammatory cells induce naïve T cells to differentiate into Th2 cells following TCR stimulation by antigen-presenting cells. The antigen-specific Th2 cells and follicular helper T cells (Tfh) induce class-switch recombination of B cells to IgE-producing B cells and plasma cells. The antigen-specific IgE binds to FcεRI receptors on mast cell and basophils. Allergens from pollen cross tight junctions and bind to corresponding antigen-specific IgE, causing the release of inflammatory mediators, such as histamine and leukotrienes from effector cells. LTP, lipid transfer protein; IFR, isoflavone reductase-like protein; TSLP, thymic stromal lymphopoietin; DC, dendritic cell; Th0, naïve T cell; LTs, leukotrienes; ILC2, group 2 innate lymphoid cell; ECP, eosinophil cationic protein; MBP, major basic protein; Tfh, follicular helper T cell.

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compared with those of food sensitized conventional WDEAI patients after ingestion of natural wheat product.[23]

These forms of type I allergy are the result of antigen-sensitization following disruption of epithelial cells by physical, enzymatic, and chemical stimuli. Pollen extract shows serine and/or cysteine endopeptidase activity although the content and releasability of the proteases differed according to the plant families.[24] Disruption of skin barrier by pollen-derived proteases may contribute to sensitization by pollen especially in peak pollen season. Many atopic dermatitis patients shows symptom flare in peak pollen season for Japanese cedar and cypress in Japan. This symptom flare in the peak pollen season occurs independently on symptoms for the pollinosis.[25] Pollen encountered damaged skin barrier invoke inflammation and may contribute to epicutaneous sensitization of pollen-derived allergens.

Japanese cedar pollen allergens

The full spectrum of allergens from JCP reacted with IgE in plasma samples obtained from patients with Japanese cedar pollinosis was analyzed using two-dimensional (2-D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting methods. This kind of comprehensive omics analysis of component allergens combined 2-D immunoblotting with subsequent protein identification by protein sequencing or mass spectrometry is known as allergenome or allergome analysis. Allergenomic analysis showed at least 131 individual spots had capacity to react with IgE in the plasma of patients with allergies to JCP.[26] To date, eight allergens have been isolated and their biological properties characterized (Table 1). Allergens are formally named using the systematic nomenclature of the Allergen Nomenclature Sub-Committee of the World Health Organization and International Union of Immunological Societies. The system uses the first three letters of a genus, a single letter for the species, and a number that refers to the chronological order of allergen purification and sometimes to the properties of the allergens.[27] Therefore, the registered allergens from JCP are designated "Cry j" from the genus *Cryptomeria* and the species *japonica*, which is the botanical name for Japanese cedar.

Cry j 1 is major component of JCP extract and most patients suffered from Japanese cedar pollinosis have specific IgG and IgE to Cry j 1. Allergenomic analysis of JCP showed Cry j 1 had highest IgE-binding frequency with patients allergic to JCP and 31 spots had higher IgE-binding capacity than that of an Cry j 2 isoform.[26] Some new JCP antigen spots were characterized as additional major allergens that have comparable IgE-binding frequency with that of Cry j 2. The IgE-binding frequency of some allergens in JCP including Cry j 1 and Cry j 2 may be underestimated on 2-D immunoblot because some conformational epitopes are not stable for heat, high and low pH condition, and denature by chemicals. It will be important to quantify the amount of each allergen in JCP and to evaluate the allergenicity by measurement of IgE reactivity or intradermal injection test using each native allergen for clarify the clinical importance of each allergen. As we pointed out above, JCP contains potential important allergen molecules other than Cry j 1 and Cry j 2. Those new JCP allergens cross-react with other plant allergens, and show sequence identity with other causative allergens identified in food allergy and pollinosis. Below we summarize the eight JCP allergen molecules identified thus far (Table 1).

Cry j 1: pectate lyase

Cry j 1, the first allergen isolated from JCP, was originally reported as sugi basic protein (SBP; Sugi means Japanese cedar in Japanese) in 1983,[28] and is a major component of JCP extract; approximately 35 mg per 100 g pollen is routinely extracted.[29] Cry j 1 is a basic glycoprotein homologous to pectate lyase and has a molecular weight of 45–51 kDa with a pI 8.9 to 9.2.[30] It is one of the major causative allergens for Japanese cedar pollinosis and its IgE-binding frequency is reported to be 86% (12/14) by enzyme-linked immunosorbent assay (ELISA) and 51% (35/68) by immunoblot analysis.[31,32] Most IgE-binding epitopes in Cry j 1 are reported to be conformational epitopes and heat-labile.[28,33] Cry j 1 exists as several isoforms in JCP that differ in their primary sequences and post-translational modification with oligosaccharides.[30] Three potential N-glycosylation sites exist in Cry j 1, and Asn-170 and Asn-333 were found to carry asparagine-linked oligosaccharides.[34] In allergenomic analysis, at least 12 Cry j 1 isoforms were fractionated on 2-D SDS-PAGE, with IgE-binding reactivity ranging from 27.5% to 75%.[26,35] The Japanese Society of Allergology standardized JCP extract as vaccines for allergen-specific immunotherapy (SIT) and the measurement of Cry j 1 content has been used as the *in vitro*

Table 1
Identified allergens from Japanese cedar pollen.

Name in original paper	Biological or perspective function	Molecular weight (SDS-PAGE, kDa)	Accession number(s) on DNA data bank of Japan (DDBJ)	Homologous allergens
Cry j 1	Pectate lyase	45-50	D34639, D26544 (Cry j IA), D26545 (Cry j IB), AB081309 (Cry j 1.1), AB081310 (Cry j 1.2)	Cha o 1 (Japanese cypress) Cup a 1 (Arizona cypress) Jun a 1 (Mountain cedar) Jun v 1 (Eastern red cedar)
Cry j 2	Polygalacturonase	37 (non-reduced) 45 (reduced)	D37765, D29772, E09607, E10716, AB081403 (Cry j 2.1), AB081404(Cry j 2.2), AB081405 (Cry j 2.3)	Cha o 2 (Japanese cypress) Cup a 2 (Arizona cypress) Jun a 2 (Mountain cedar) Jun v 2 (Eastern red cedar)
Cry j 3	Thaumatin-like protein	19 (non-reduced) 27 (reduced)	AB081303 (Cry j 3.1), AB081304 (Cry j 3.2), AB081305 (Cry j 3.3), AB186384 (Cry j 3.4), AB186385 (Cry j 3.5), AB186386 (Cry j 3.6), AB212218 (Cry j 3.7), AB254807 (Cry j 3.8)	Jun a 3 (Mountain cedar) Jun v 3 (Eastern red cedar) Mal d 2 (Apple) Pru av 2 (Cherry)
CJP-4	Class IV chitinase	34	AB196451	Pers a 1 (Avocado) Cas s 5 (Chestnut) Mus a 1 (Banana)
CJP-6	Isoflavone reductase-like	34 (recombinant)	AY028631	Bet v 5 (Birch), Pyr c 5 (Pear)
CJP-8	Lipid transfer protein	20 (recombinant)	AB520844	Pru p 3 (Peach), Mal d 3 (Apple), Fra a 3 (Strawberry)
CPA9	Subtilisin-like serine protease	90	Not registered	Cuc m 1 (Melon)
CPA63	Aspartic protease	52 (recombinant, proenzyme) 42 (mature enzyme)	AB510538	Not reported

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surrogate assay for standardization of the JCP extract in Japan.[36,37] Pectate lyase is a common component in several species of plant pollens; therefore, the Cry j 1-specific IgE have the potential to cross-react with other pectate lyase allergens from tree pollens such as Chao 1 (78% sequence identity with Cry j 1), Bet v 1 (20%), Car b 1 (14%), and Aln g 1 (18%) from Japanese cypress (*Chamaecyparis obtusa*), birch (*Betula verrucosa*), hornbeam (*Carpinus betulus*), and alder (*Alnus glutinosa*), respectively.[8]

Cry j 2: polygalacturonase

Cry j 2, which was the second allergen isolated from JCP, was also reported to be a major allergen,[31] with 2.9–14 mg extracted per 100 g pollen, depending on the extraction method ([29] and unpublished data). Cry j 2 is a basic protein homologous to polygalacturonase, with a molecular weight of 45 kDa under reduced conditions.[31,38] Its IgE-binding frequency is reported to be 71% (10/14) by ELISA and 47% (32/68) by immunoblot analysis.[31,32] Cry j 2 has one potential N-glycosylation site, although its actual glycosylation remains to be confirmed. Allergenomic analysis revealed at least three Cry j 2 isoforms with different isoelectric points. There was no marked difference in the IgE-binding reactivity among the different isoforms (range 32.5%–40%) in Japanese cedar pollinosis patients.[26] Cry j 2-specific IgE showed cross-reactivity with Chao 2 (74% sequence identity with Cry j 2), another polygalacturonase allergen from Japanese cypress pollen.[39]

Cry j 3: thaumatin-like protein

Cry j 3, which was isolated from a cDNA library derived from JCP, is a homologue of Jun a 3, a major allergen from mountain cedar (*Juniperus ashei*) pollen.[40] Clones encoding Cry j 3 were isolated from Cry j 3.1 to Cry j 3.6.[41] Native Cry j 3 was isolated from JCP using multi-dimensional chromatography, and the corresponding clone was designated Cry j 3.8.[42] Native Cry j 3 is glycoprotein with a molecular weight of 27 kDa under reduced conditions and the IgE-binding frequency is 27% (27/100) by ELISA. Cross-reactivity between Cry j 3 and Jun a 3 (86% sequence identity with Cry j 3.8) was expected because three sequential epitopes of Jun a 3 are highly conserved in Cry j 3.8, with identities of 92%, 100%, and 86%. Rabbit antiserum raised against Jun a 3 recognized Cry j 3.[42,43] Cry j 3 is a homologous allergen to thaumatin-like proteins (TLPs) and belongs to the pathogenesis-related-5 (PR-5) group family. Thaumatin is an intensely sweet protein that is isolated from the fruits of a West African rain forest shrub (*Thaumatococcus daniellii*) and is induced in response to infections by pathogens and environmental factors.[44] PR family proteins are reported as plant pan-allergens. Cry j 3 is also homologous to other PR-5 member allergens such as Mal d 2 (45%), Pru av 2 (44%), Act d 2 (50%), and Cap a 1 (47%) from apple (*Malus domestica*), sweet cherry (*Prunus avium*), kiwi fruit (*Actinidia deliciosa*), and bell pepper (*Capsicum annuum*), respectively. Cry j 3 is implicated in cross-reactivity between allergies to JCP and tomato (*Solanum lycopersicum*) because two isoforms of TLP (NP24, 44%) are isolated from tomato fruit.[45,46] Therefore, it can be speculated that Cry j 3 is important in oral allergy syndrome (OAS) among patients with Japanese cedar pollinosis and allergies to fruit and vegetables.

CJP-4: class IV chitinase

CJP-4 is a class IV chitinase identified in allergenomic analysis of JCP.[47] It is a 34-kDa protein with endochitinase activity. The IgE-binding frequency of CJP-4 is comparable with that of Cry j 1, although the amount of CJP-4 is much less than that of Cry j 1. CJP-4 is a class IV chitinase categorized into family 19 chitinases, which possess two consensus family 19 signature motifs in the catalytic domain, in addition to the chitin-binding motif in the hevein domain. Endogenous acidic mammalian chitinase was reported to exacerbate Th2 inflammation and airway responsiveness, in part by accelerating IL-13 pathway activation and chemokine induction.[48] Exogenous pollinic chitinase may also have the potential to participate in these physiological functions to enhance Th2 responses against JCP allergens. CJP-4 is a member of the PR-3 family and is homologous to PR-3 allergens, such as Hev b 11 (38.4% sequence identity with CJP-4), Pers a 1 (38.2%), Cas s 5 (38.1%), and Mus a 1 (41.5%) from latex (*Hevea brasiliensis*), avocado (*Persea americana*), chestnut (*Castanea sativa*), and banana (*Musa acuminata*), respectively. CJP-4 has a highly conserved chitin-binding motif and a hevein-like domain responsible for the cross-reactivity in latex-fruit syndrome. CJP-4 cross-reacts with IgE from patients allergic to latex and inhibits binding between the latex extract and the IgE. Therefore, CJP-4 may be important as a cross-reactive allergen in latex-fruit syndrome.

CJP-6: isoflavone reductase-like protein

CJP-6 was cloned as an isoflavone reductase-like allergen from a JCP cDNA library. Native (n)CJP-6 has not been isolated and its immunological properties remain to be elucidated; however, recombinant (r)CJP-6 exhibited 76% IgE-binding frequency (19/25) in Cry j 1-positive patients with Japanese cedar pollinosis.[49] CJP-6 is homologous to other members of the isoflavone reductase family, such as Bet v 5 (61% sequence identity with CJP-6) and Pyr c 5 (60%) from birch and pear (*Pyrus communis*), respectively; therefore, it is also important to test whether the CJP-6 molecule shows IgE cross-reactivity with isoflavone reductase counterparts from other pollens and plant-based foods.

CJP-8: lipid transfer protein homologue

The other allergens, CJP-8, CPA9, and CPA63 were also identified in the allergenome analysis. CJP-8 is homologous to lipid transfer protein (LTP).[50] Although nCJP-8 has not been isolated, rCJP-8 exhibited 37.5% IgE-binding frequency (6/16) in patients with Japanese cedar pollinosis. The LTP allergens, rPar j 1 (11% sequence identity with CJP-8) from pellitory (*Parietaria judaica*) and rPru p 3 (15%) from peach (*Prunus persica*), inhibited the binding between rCJP8 and rCJP8-positive JCP-allergic plasma IgE, indicating that CJP8 is also a pan-allergen for OAS.

CPA9: subtilisin-like serine protease

CPA9 is a plant subtilisin-like serine protease and nCPA9 reacted with 88.5% (23/26) of patients allergic to JCP in ELISA.[51] Serine proteases are known as house dust mite (*Dermatophagoides farinae* and *D. pteronyssinus*) allergens and three classes have been reported as allergens; group 3 (trypsin), group 6 (chymotrypsin), and group 9 (collagenase). Serine protease allergens, Pen ch 13 (alkaline serine protease) and Pen ch 18 (vacuolar serine protease), are also reported in allergenic fungi (*Penicillium chrysogenum*). CPA9 is homologous to Cuc m 1 (40% sequence identity with CPA9) in muskmelon (*Cucumis melo*) and melon extract inhibits binding between nCPA9 and CPA9-specific IgE. Therefore, CPA9 is also implicated as a pan-allergen for OAS.

CPA63: aspartic protease

CPA63 structurally belongs to atypical type of plant aspartic protease family members that shows proteolytic activity under acidic conditions.[52] rCPA63 was shown to react with IgE from 58%

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(18/31) of patients allergic to JCP by ELISA. rCPA63 was identified as a 52-kDa proenzyme of a 42-kDa mature enzyme generated by autolysis. nCPA63 appeared as a 42-kDa protein in 2-D SDS-PAGE, demonstrating that nCPA63 is present in the mature form in JCP. Another aspartic protease allergen, Bla g 2, was identified in the German cockroach (*Blattella germanica)*.

Impact of component-resolved diagnosis on Japanese cedar pollinosis

It will be important to analyze the IgE-binding reactivity and intensity to group 1 and 2 major allergens in cedar/cypress species and pan allergens for plant-fruit syndrome to discriminate Japanese cedar pollinosis, other cypress/juniper pollinosis, and OAS. It is necessary to confirm strong IgE reactivity with Cry j 1 and Cry j 2 and weak or negative reactivity with major allergens from other cypress and juniper to conclude that patients are sensitized to Japanese cedar pollen. Strong IgE-reaction with CJP-4, CJP-6, CJP-8, or CPA9 may suggest that patients would be suffered OAS rather than Japanese cedar pollinosis. Further information will be necessary to achieve more accurate diagnosis for Japanese cedar pollinosis by component-resolved diagnosis (CRD).

Allergen-specific immunotherapy

Allergen-specific immunotherapy (SIT) is considered to be the only curative intervention associated with changes of the natural course of IgE-dependent type I allergy. The first report of SIT involved injection immunotherapy for hay fever without an adjuvant in 1911.[53] During the century that followed, the protocol for SIT has improved to increase efficacy and safety through coadministration of allergens with immunostimulatory adjuvants, premedication with anti-histamine or anti-human IgE antibodies, or administration of allergens via alternative routes.[54] Over the last two decades, sublingual SIT (SLIT) has become recognized as a preferable form of immunotherapy compared with conventional subcutaneous SIT (SCIT) in terms of safety, feasibility, and convenience for patients.[55]

Crude extracts from natural allergen sources have been used as SIT vaccines widely in the clinics. The World Allergy Organization recommends that standardized vaccines should be used for SIT if they are available.[56] Over the last 20 years, many suppliers have standardized their allergen extracts based on the concentration of major allergens or bioactivity in intradermal injection tests using individual in-house reference materials. However, due to differences in the protocols and methods for standardization of allergen extracts used by different suppliers, it is still difficult to compare the therapeutic effects and safety among clinical trials involving different allergen extracts. It has been proposed that vaccines should be standardized using a protocol based on mass units of major allergens and that the active ingredients of the treatment should be quantified. Isolation and characterization of component allergens in allergen sources is still an important issue for the purposes of quantifying the active ingredients in an extract.

Polyclonal IgE antibodies from an allergic patient react with many allergens in a single allergen source and the profiles or IgE-reactive allergens differ among individuals. In the allergenome study of JCP, the average number of IgE-reactive spots in 40 symptomatic patients with JCP allergy was 37 ± 22 (range, 4–86) spots. Among these patients, 45% (18/40) showed IgE-binding spectra including reactivity (34 ± 17 spots) with many allergens other than the major allergens, Cry j 1 and Cry j 2. A further 25% (10/40) showed spectra that were predominantly the result of reactivity with the major allergens (34 ± 14 spots). In addition, 17.5% (7/40) showed strong reactivity with numerous allergens including the major allergens (63 ± 15 spots) and finally 12.5% (5/49) showed weak reactivity with a few allergens (6 ± 1 spots).[26] This report did not include details of OAS status; however, all patients showed a positive RAST score for JCP (RAST score ≥2) and would be candidates for SIT using JCP extract. These differences in IgE-spectra among individuals may represent the basis of an approach to distinguish good and poor responders for SIT.

Two years of SLIT treatment for Japanese cedar pollinosis significantly ameliorated nasal and ocular symptoms and reduced the requirement for medicines in the second year; this amelioration was sustained 1 year after termination of the treatment. This report implicated induced regulatory T cells (defined as a IL-10$^+$ Foxp3$^+$ T cells in the population of CD25$^+$ CD4$^+$ leukocytes) as biomarkers for monitoring the response to SIT, and furthermore, indicated that the ratio of specific IgE to total IgE may be a candidate predictive biomarker to distinguish good and poor responders prior to treatment.[57] Thus, it can be speculated that a combination of IgE-binding allergen profiling and the ratio of specific IgE to total IgE may be a good predictive biomarker of the efficacy of SIT.

Impacts of component-resolved diagnosis on allergen-specific immunotherapy

SIT is an antigen-specific treatment; therefore, accurate diagnosis is highly important.[58] Accurate elucidation of the sensitizing allergen source for individual patients is a first step to achieving effective immunotherapy, although allergic patients are often polysensitized with pollens from many plant species and other sources, including vegetables, fruits, mites, fungi, and animals. Discrimination of pollinosis and food allergy, especially allergy to vegetables and fruits, is important to determine allergens for SIT. CRD is a powerful method for the determination of the sensitizing allergen source. Simultaneous analysis of IgE reactivity with allergens, including major allergens from various pollen species and pan-allergens such as non-specific lipid transfer protein and profilin is necessary to discriminate pollinosis from OAS.[59] Construction of a database of patterns of IgE-reactive allergens and variations in sensitizing allergen sources in various geographic areas may be useful for such discrimination and selection of allergens for SIT in the clinic.

This kind of serological approach using plasma IgE is usually easy, low-cost, and safe compared with *in vivo* allergen provocation tests; however, IgE serology is not always a sensitive predictor of allergenic activity and clinical relevance. A study that compared the IgE reactivity to recombinant component allergens from timothy (*Phleum pratense*) and birch with skin reactivity showed a strong association between a positive skin reaction and the presence of allergen-specific IgE, although the correlation with allergen-specific IgE levels was weak.[60] Native allergens contain various isoforms with minor sequence variations or post-translational modifications, and these differences may alter the IgE-binding capacity of each isoform.[26,61] Therefore, the recombinant allergens to be used for *in vitro* or *in vivo* diagnosis should contain most of the IgE epitopes present in natural allergens.

Selection of patients and allergens for allergen-specific immunotherapy

CRD also has an impact on exclusion of patients from SIT. A subpopulation of patients who report symptoms of pollinosis were originally sensitized with plant foods and have specific IgE that react strongly with pan-allergens but weakly with the major pollen allergens. The application of CRD to select appropriate patients for SIT is called CRD-based immunotherapy (CRD-IT). In a study conducted in 651 children with moderate-to-severe pollen-related

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rhinitis in Italy, 10%, 28%, and 30% of patients with positive skin prick test (SPT) reactions against clinically relevant sensitization with grass (*P. pratense)*, olive (*Olea europaea*), and pellitory pollen extracts, respectively, did not show IgE reactivity with the major allergens Phl p 1, Phl p 5, Ole e 1, or Par j 2. In the case of birch and mugwort (*Artemisia vulgaris*) pollinosis, more than 50% of the patients with positive SPT reactions against each extract showed negative IgE reactivity to Bet v 1 and Art v 1, respectively. The population with inconsistencies between the results of SPT and CRD showed IgE sensitization to profilin (Phl p 12) or polcalcin (Phl p 7), although this was not true for all patients. This study showed that among 508 of the 651 patients who would have received an SIT prescription when evaluated only on the basis of clinical history, pollen calendars, and SPT responses, 170 (33%) would have received SIT with a different composition, and a further 52 (10%) would have received no SIT at all.[62] CRD also reveals a mismatch or over/underpowered immunization by extract for SIT. A SIT study using an allergen cocktail for *P. pratense* (Phl p 1, Phl p 2, Phl p 5, and Phl p 6) classified patients into four categories based on the patient's profile IgE reactivity with the allergens. Among the 176 patients included, only 4% were matched for IgE profile and components of the extract; of those showing the same profile between IgE-reactive allergens and the components of the extract. Among the patients, 29% were underpowered; with a greater number of IgE-reactive allergens than the number of components. A further 32% were overpowered; with fewer IgE-reactive allergens than the number of components. In addition, 32% were over/underpowered; with greater numbers of IgE-reactive allergens than the number of components and the extract contained allergens to which the patient was not sensitized, and finally 3% were completely mismatched.[63] Therefore, CRD is considered to be useful for precise selection of an allergen source or allergen cocktails for effective SIT; however, further clinical investigations are required to verify the benefit of CRD-IT in achieving more effective SIT compared with classical SIT based on a clinical history, positive IgE reactions to whole extracts, and positive SPT reactions.

Prediction of adverse events by component-resolved diagnosis

The use of CRD in SIT may also be valuable in predicting the occurrence of adverse events in SLIT. These are usually very mild and involve local reactions such as itching and swelling of the oral mucosa,[64] which may be triggered partially by pan-allergens for latex-fruit syndrome and OAS, such as chitinase and LTP, contained in pollen extracts.[47,65] Patients exhibiting strong IgE reactivity with these pan-allergens are likely to show local side-effects induced by pollen SLIT, and the CRD profile may be useful in predicting local or gastrointestinal side-effects as a result of SLIT using whole-pollen extract.

Therapeutic effects of allergen-specific immunotherapy on reactions to homologous plant species

Many allergens commonly contained in plants show IgE cross-reactivity and the eliciting allergen is sometimes different from the original sensitizing allergen source. This type of cross-reactivity observed in B cell epitopes reflects a repertoire of immunoglobulin specificity. Such cross-reactivity in T cell recognitions remains to be elucidated and the differences in T cell recognition among homologous allergens may lead to the differences in the therapeutic effects of SIT. SIT using JCP extract did not improve the clinical symptoms of Japanese cypress pollinosis, although the amino acid sequences of the major Japanese cedar allergens are highly homologous to those from cypress.[66] Another report also indicated poor clinical responses to SIT using short ragweed (*Ambrosia artemisiifolia*) pollen extract for giant ragweed (*A. trifida*) pollinosis patients, and *vice versa*.[67] In a case of OAS, SIT using birch pollen extract was not effective for already established apple allergy, although the major allergens, Bet v 1 in birch and Mal d 1 in apple, show 64% identity at the amino acid level.[68] Very small differences in amino acid sequences lead to distinct T cell responses and may affect the induction of T cell tolerance or regulatory T cells by SIT.[69] Thus, the geographic situation and species of scattering pollen should be considered carefully in the selection of allergens for SIT.

Personalized allergen-specific immunotherapy

CRD facilitates profiling of IgE-reactive allergens for individuals. It is theoretically possible to personalize the extract used for SIT such that it contains only allergens to which the patient is sensitized; this is known as personalized medicine or tailor-made therapy.[70] However, in practice, this requires the preparation of an extract containing every component as a recombinant or a purified native allergen in a process that complies with good manufacturing practice suitable for medical use. Consequently, this approach has poor feasibility in terms of practicality, cost, and solubility of the recombinant proteins. Depending on the allergen source, it may be possible to achieve clinical efficacy using one major allergen that is comparable with that achieved using a whole extract. A clinical trial of SCIT with pullulan-conjugated Cry j 1 for Japanese cedar pollinosis showed that the improvement in clinical symptoms after pullulan-Cry j 1 administration was comparable with that observed in response to the whole-pollen extract, with fewer adverse events.[71] This effect was also observed for patients with birch pollinosis. In a study using rBet v 1, nBet v 1, birch pollen extract, and placebo for SIT, significant reductions in rhino-conjunctivitis scores, rescue medication scores, and skin sensitivities were observed in the rBet v 1, nBet v 1, and extract groups compared with the placebo group, and the degree of improvement was comparable among the three actively treated groups.[72] The number of allergens included in a SIT vaccine to achieve a therapeutic effect comparable with that achieved with an extract may differ among species of allergen sources. For instance, it appears that there are fewer allergens from tree pollen than from grass pollen.[60] The profile of IgE-reactive allergens for tree is apparently uncomplicated and comprises predominantly group 1 and 2 major allergens; it can be speculated that this accounts for the achievement of comparable clinical improvement using a single major allergen in SIT to that achieved with whole extract from Japanese cedar and birch pollen. Grass pollen contains a large number of IgE-reactive major allergens, therefore, only one or a combination of a few allergens may not be enough to achieve clinical improvement comparable with that achieved with the whole extract.

De novo sensitization during SIT is also an important issue in considering the advantage of CRD-IT. It will be very difficult to distinguish whether *de novo* sensitization occurred during SIT treatment or by natural exposure to allergens. In a study of successful rush SCIT for up to 3 years in patients of birch pollinosis, 17 of 26 patients (65%) showed new IgE specificities after SCIT compared with those detected before SCIT based on immunoblot analysis. This new sensitization was observed during the early phase of rush SCIT including during the first birch season in three of the patients, while no patients without SCIT showed new IgE sensitization during the study.[73] A study of SCIT for timothy grass pollinosis also showed *de novo* sensitization to allergens that were not recognized before treatment on the basis of Pharmacia CAP analysis. During SIT in a total of eight patients, one showed *de novo* sensitization to Phl p 4 and Phl p 6, while another was transiently sensitized to Phl p 4.[74] These reports showed a risk of *de novo* sensitization by SIT using a natural extract. However, a study of

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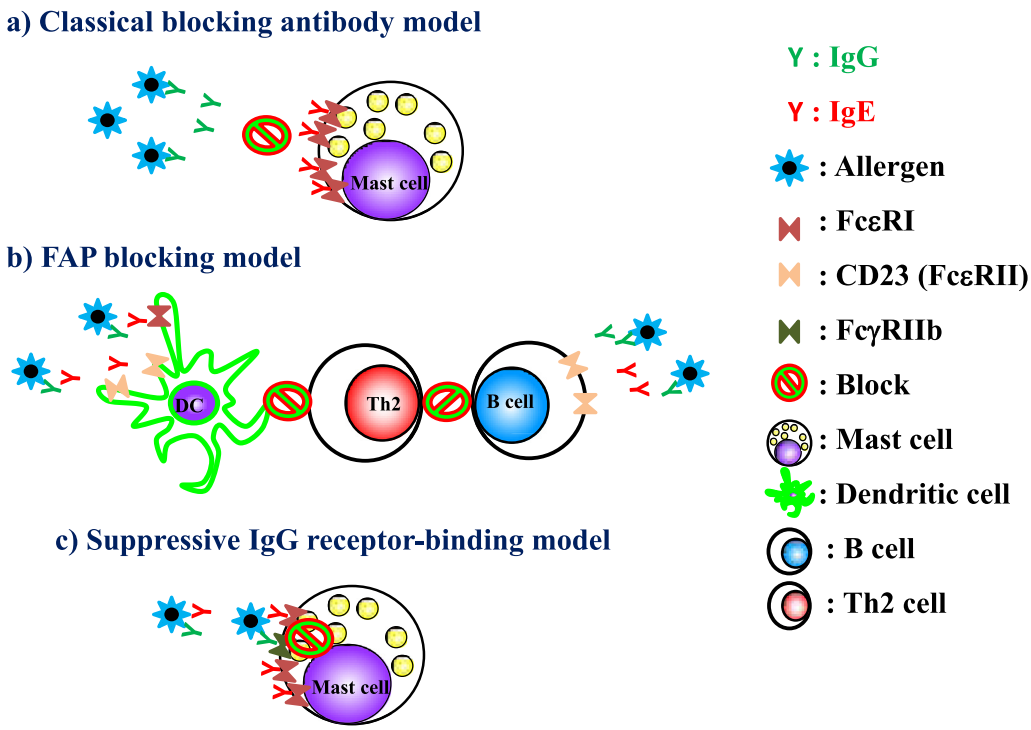

Fig. 2. Theoretical models of the mechanism by which IgG suppresses the activation of Th2 or inflammatory cells during allergen-specific immunotherapy (SIT). a) IgG or IgG4, which recognize the same epitope as IgE, prevent binding of IgE and the invading allergen. The blocking IgG antibody prevents allergen binding to IgE on the surface of mast cells. b) IgG or IgG4, which recognize the same epitope as IgE, bind to allergens preventing the formation of immunocomplexes between allergens and IgE, and prevent subsequent IgE-facilitated antigen presentation (FAP; IgE-mediated uptake by antigen-presenting cells). The blocking IgG prevents FAP by blocking the binding of immunocomplex with CD23 (FcεRII) on B cells, and with CD23 and FcεRI on dendritic cells. c) A subtype of IgG, which recognizes an epitope distinct from that bound by IgE, binds allergen independently to form immunocomplexes consisting of IgG, IgE, and allergen. The IgG bound to the allergen binds to suppressive Fcγ receptors, such as FcγRIIb, and inhibits activation signals from FcεRI.

cluster SCIT for timothy grass pollinosis using the pollen extract did not show *de novo* sensitization to new components, including rPhl p 1, rPhl p 2, nPhl p 4, rPhl p 5, rPhl p 6, rPhl p 7, rPhl p 11, or rPhl p 12 as determined by CAP analysis in 33 patients after 15 weeks of SCIT injections. The SCIT significantly increased antigen-specific IgG4 production and the produced IgG4 recognized only the components recognized by IgE before the treatment. Patients lacking IgE reactivity to individual components before SCIT did not produce specific IgG4 to the same component or produced negligible amounts.[75] A randomized double-blinded, placebo-controlled, multicenter phase II/III study for timothy pollinosis also showed no additional *de novo* sensitization during short-course SLIT treatment by CAP analysis of rPhl p 1, rPhl p 5, rPhl p 7, and rPhl p 12.[76] The absence of *de novo* sensitization during SLIT was also reported in a study conducted in children with timothy grass pollinosis.[77] Considering the results of these studies, the risk of *de novo* sensitization during SIT using crude extract remains unclear, although the risk associated with SLIT appears to be low. The induction of IgG and IgG4 reactive to allergens recognized by IgE prior to treatment may be a key factor in achieving clinically successful SIT[78] (Fig. 2).

Personalized allergen-specific immunotherapy for Japanese cedar pollinosis

SIT using modified Cry j 1 significantly improved symptoms for Japanese cedar pollinosis in patients diagnosed by clinical history and positive IgE to crude extract of JCP.[71,79] Using Cry j 1 will be sufficient to achieve clinical improvement for Japanese cedar pollinosis in patients diagnosed more accurately by CRD. For the patients who do not show clinical improvement using Cry j 1, it will be considered to be add other allergens for SIT vaccine based of the information of reactive allergens elucidated by CRD.

Conclusions

Although the immunochemical properties of the full spectrum of the major and important allergens of JCP are not yet fully understood, the accumulating information on the molecular properties of these allergens is important in elucidating their crucial roles in sensitization. The application of this information in combination with molecular biotechnological and immunochemical techniques will facilitate the preparation of cocktails of allergens formulated with predetermined and uniform allergen levels. The availability of these allergens could increase the feasibility of the development of innovative, patient-based tests for CRD, and individualized SIT.

In this review, we report the analysis of component allergens and clinical trials mainly in the context of Japanese cedar pollinosis, which is a typical type I seasonal allergy for which the causative allergens are well characterized. In our opinion, Japanese cedar pollinosis is a good model for the evaluation of novel diagnosis methods, therapeutic vaccines, and the sensitizing and tolerance-inducing mechanisms of SIT against the background of genetics and lifestyle in Japan.

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Conflict of interest

TF belonged to a co-operative laboratory in RIKEN with Torii Pharmaceutical Co., Ltd. to develop a vaccine for allergen-specific immunotherapy for Japanese cedar pollinosis from May 2010 to March 2015. SK has no conflict of interest.

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