Japanese Cedar Pollen-Based Subcutaneous Immunotherapy Decreases Tomato Fruit-Specific Basophil Activation

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Key Words
Basophil activation · Flow cytometry · Japanese cedar pollinosis · Specific immunotherapy · Pollen-food allergy syndrome · Oral allergy syndrome

Abstract
Background: Some patients with Japanese cedar pollen (JCP)-induced allergic rhinitis develop pollen-food allergy syndrome (PFAS) as a reaction to tomato fruit. Pollen allergen-specific subcutaneous immunotherapy (SCIT) is reportedly beneficial for some associated food allergies; however, the reported changes in food allergen-specific immunoglobulin (IgE) and IgG4 levels are inconsistent. Here, we investigated immunologic reactivity to tomato fruit after JCP-based SCIT.

Methods: Twenty-three children (aged 6–17 years) with JCP-induced allergic rhinitis and sensitized to tomato (serum tomato fruit-specific IgE level >0.34 UA/ml) received JCP-based SCIT. Basophil activation by tomato and JCP extracts and serum-specific IgE and IgG4 levels against these allergens were determined before and after 4 or 5 months of maintenance SCIT. Basophil activation was assessed by monitoring CD203c upregulation on flow cytometry. Results: JCP-based SCIT significantly reduced the basophil activation caused by tomato fruit (p = 0.03) and JCP (p < 0.001) extracts. JCP-specific IgG4 levels markedly increased after SCIT (p < 0.001), whereas tomato fruit-specific IgG4 levels did not. After SCIT, no significant changes were observed in specific IgE levels for tomato fruit (p = 0.11) or JCP (p = 0.19).

Conclusions: Tomato fruit-specific basophil activation decreases after JCP-based SCIT, suggesting that it is efficacious in relieving and preventing the symptoms of PFAS in patients with JCP-induced allergic rhinitis.

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Introduction

In total, 5–8% of patients allergic to pollen develop pollen-food allergy syndrome (PFAS), which presents as itching in the mouth and throat immediately after eating fresh fruits and vegetables, which sometimes leads to angioedema. In Europe and the USA, birch pollen allergy is common whereas in Japan, Japanese cedar pollen (JCP)-induced allergic rhinitis is the most common al-
Allergic disease [1]. Some patients with JCP-induced allergic rhinitis also develop PFAS as a reaction to tomato fruit. Previously, using radioallergosorbent test inhibition, we demonstrated cross-reactivity between the allergens in tomato fruit and JCP [2]. The allergen responsible for this may be Cry j 2, i.e. the major allergen of JCP, which has a 40% similarity to polygalacturonase from tomato fruit.

Allergen-specific immunotherapy is an established treatment for pollen allergy [3]. However, there are no standard treatments for food allergies, including PFAS, other than avoiding the implicated foods. Pollen allergen-specific subcutaneous immunotherapy (SCIT) is reportedly beneficial for associated food allergies, primarily for patients with a birch pollen-induced allergy to apples [4–7]. Some studies have shown the beneficial effects of SCIT on PFAS occurring in response to foods other than those containing Bet v 1-related protein [8, 9]. However, Mordzynski and Zawisza [10] reported that it is possible to develop new allergies during pollen-based SCIT.

Evaluating reactivity to a pollen-associated food after pollen-based SCIT remains difficult. The gold standard for diagnosing an immunoglobulin (Ig)E-mediated food allergy is a double-blind, placebo-controlled food challenge. However, fresh fruits have characteristic smells and tastes, and it is difficult to blind patients to these [11]. Skin-prick testing and serum-specific IgE measurement have been used to quantitatively assess responses to pollen-associated food allergens, but these tests have poor predictive values [12]. The basophil activation test (BAT) has attracted attention for the assessment of type I allergic responses [13]. Basophil responsiveness may have a higher specificity than serum-specific IgE tests for diagnosing PFAS [12], and BAT has been used to assess the effects of SCIT [14, 15]. CD203c (ectonucleotide pyrophosphatase/phosphodiesterase) is an ectoenzyme expressed only on basophils, mast cells and their CD34-positive progenitor cells in peripheral blood, and CD203c represents a basophil activation marker [16].

Using these assays, we investigated tomato fruit-specific basophil activation after JCP-specific SCIT in patients with JCP-induced allergic rhinitis.

Material and Methods

Participants

The procedures used in this study and the possible risks were explained to all patients and their parents, and their written informed consent was obtained. This study conformed to the guidelines established by the Declaration of Helsinki and was approved by the Research Ethics Committee of the Fujita Health University, Aichi, Japan (No. 10–224).

For this prospective study, we enrolled Japanese children who exhibited symptoms of mild-to-severe JCP-induced allergic rhinitis and tomato sensitization (i.e. serum tomato fruit-specific IgE levels $>0.34$ UA/ml). A baseline immunologic evaluation, which included specific IgE and IgG$_4$ levels and basophil activation, was performed in July and August (immediately before starting SCIT) to avoid the effects of seasonal pollen exposure. Reevaluations were performed between late November and early February (before the JCP season) after 4 or 5 months of maintenance SCIT (fig. 1).

SCIT with JCP Extract

All patients underwent injection SCIT with standardized JCP extract (Torii Pharmaceutical Co., Ltd., Tokyo, Japan). The protocol used was: 0.1, 0.2, 0.6 and 1 Japanese allergy units (JAU) on day 1; 2, 6, 10 and 20 JAU on day 2; 40 and 60 JAU on days 3 and 4; 100 JAU on days 5 and 6; 140 JAU on day 7; 200 JAU on days 8 and 9. The planned maintenance dose was 200 JAU or the maximum tolerated dose to be given within 2 weeks. Treatment began at the end of July or August. Maintenance doses were given at 4-week intervals. The 200-JAU dose of the extract contained 0.06–0.25 μg/ml of Cry j 1 and 0.08–0.31 μg/ml of Cry j 2, which are the major allergens of JCP [17].

Tomato and JCP Extracts

Tomato and JCP extracts were prepared using our previously reported methods [2], with modifications. Whole, ripe tomato fruit was homogenized with extraction buffer (2.5 ml/g fresh tissue weight) that included 1 M sucrose (pH 9.5), 2% (w/v) suspended solid polyvinylpolypyrrolidone, 2 M ethylenediaminetetraacetic acid disodium salt and 10 mM sodium diethyldithiocarbamate. After stirring at 4°C for 18 h, debris was removed by centrifugation at 2,400 g for 10 min. The precipitate was collected.
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by centrifugation at 5,000 g for 15 min, and the supernatants were used after filtration through a 0.45-μm Millex-HV filter unit (EMD Millipore Corp., Billerica, Mass., USA). JCP (Allergon AB, Angelholm, Sweden) was extracted in 0.125 M NaHCO₃ (1:20 w/v) at 4 °C overnight with rotation. Extracts were centrifuged at 10,000 g at 4 °C for 10 min, and the supernatants were used after filtration through a 0.45-μm Millex-HV filter unit. Extract protein concentrations were determined using a Pierce™ bicinchoninic acid protein assay kit (Thermo-Fisher Scientific, Inc., Rockford, Ill., USA).

**Assays for IgE and IgG₄**

Patients’ serum-specific IgE and IgG₄ antibody levels were determined using the ImmunoCAP® System (Phadia AB, Uppsala, Sweden). The cut-off levels were 0.35 UA/ml for specific IgE and 0.07 mgA/l for specific IgG₄.

**Basophil Activation and Flow Cytometry Analysis of Fcε Receptor I Levels**

Basophil activation was determined using an allergenicity kit (Beckman Coulter Inc., Fullerton, Calif., USA), according to the manufacturer’s instructions. All assays used whole fresh blood within 24 h of sampling. Briefly, heparin-anticoagulated peripheral blood samples were incubated at 37°C for 15 min with fluorescein isothiocyanate-labeled anti-CRTH2, phycoerythrin-labeled anti-CD203c and phycoerythrin-cyanine 7-labeled anti-CD3 monoclonal antibodies in the presence of allergen or antigen.

Phosphate-buffered saline and anti-IgE antibodies (10 μg/ml) were used as negative and positive controls, respectively. Samples were analyzed using a FACSCalibur™ cell analyzer with CellQuest™ software (Becton, Dickinson and Company, Franklin Lakes, N.J., USA). Basophils were identified based on their forward- and side-scatter properties, the absence of CD3 expression and the presence of CRTH2 expression (fig. 2).

To assess allergen-specific basophil activation, we used a series of concentrations of tomato fruit (4 concentrations, range: 10,000–10 μg/ml total protein) or JCP (5 concentrations, range: 10–0.001 μg/ml total protein) extracts. Data acquisition was generally for 500 basophils, and we excluded samples for which data acquisition was for <200 cells.

We used fluorescein isothiocyanate-conjugated anti-human Fc receptor I (FceRI) α-chain antibody CRA1 (Cosmo Bio Co., Ltd., Tokyo, Japan) and anti-IgE antibodies. Basophil FceRI expression in mean fluorescence intensity (MFI) units was determined after gating the basophil population (fig. 3). We used 500 basophils for FceRI data acquisition.

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and Immunoblotting**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 4–12% NuPAGE® Novex® Bis-Tris Mini Gels (Life Technologies, Inc., Carlsbad, Calif., USA) according to the Laemmli method [18] under reducing conditions. Tomato extracts (45 μg/ml) were separated using 4-morpho-
linepropanesulfonic acid buffer at a constant 200 V for 50 min. After SDS-PAGE, proteins were transferred to Immobilon® P Membranes (EMD Millipore) as previously reported [19] and blocked with SuperBlock® Blocking Buffer (Thermo-Fisher Scientific) in Tris-buffered saline. We added serum obtained before and after treatment to the membrane and incubated it overnight. Serum from a patient with JCP-induced allergic rhinitis without tomato sensitization was used as a negative control. After washing the membranes, we added phosphatase-labeled goat anti-human IgE antibody (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md., USA) diluted 1:2,000, and visualized IgE using nitroblue tetrazolium/5-bromo-4-chloro-3′-indolyphosphate solution (Kirkegaard and Perry Laboratories, Inc.) as a coloring agent.

Statistical Analysis
We established dose-response curves for basophil activation based on 10-fold decreasing concentrations of the respective extracts. We used two-way analysis of variance (ANOVA) for the dose-response curve. Furthermore, basophil activation was assessed from the area under the dose-response curve (AUC). Results for nonnormally distributed continuous variables were expressed as medians and interquartile ranges, and before and after treatment results were compared using the Wilcoxon matched-pairs signed-rank test. p < 0.05 was considered significant. Data were excluded if the paired before- and after-treatment blood samples were unavailable. Statistical analysis was performed using GraphPad Prism 6.01 (GraphPad Software Inc., San Diego, Calif., USA).

Results

Study Population Characteristics
A total of 23 patients participated in this study, i.e. 19 boys and 4 girls, with a median age of 10.8 years (range 6.5–17 years). Their median tomato fruit–specific IgE level was 1.84 UA/ml (range 0.43–12.6 UA/ml) and median JCP-specific IgE level was 39.4 UA/ml (range 2.86–1,709.0 UA/ml).

Five patients (21.7%) experienced itching of the throat and lip angioedema after ingesting tomato fruit; however, they did not experience any systemic symptoms such as urticaria, coughing, wheezing or abdominal pain. The remaining patients did not experience any symptoms after ingesting tomato fruit. After SCIT, none of the patients developed any new food allergies. Among those with PFAS in response to tomato fruit, one was able to eat tomato fruit without symptoms. However, the others with PFAS refused to eat tomato fruit.

SCIT with JCP Extract Reduces Basophil Responsiveness to in vitro Stimulation with Tomato and JCP Extracts
Basophils were activated by the tomato and JCP extracts in a dose-dependent manner in 22 patients. Both of the activations were dose-dependent, and the dose-response curves shifted downward after SCIT (fig. 4). Following activation by tomato extract, two-way ANOVA showed a significant effect of treatment (F(1, 144) = 6.543; p = 0.0116) and concentration (F(3, 144) = 58.15; p < 0.0001). In addition, the treatment-by-concentration interaction was not significant (F(3, 144) = 11.72; p < 0.0001; fig. 4a). Following activation by JCP extract, two-way ANOVA showed a significant effect of treatment (F(1, 21) = 29.02; p < 0.0001) and concentration (F(4, 84) = 112.5; p < 0.0001). In addition, the treatment-by-concentration interaction was significant (F(4, 84) = 11.72; p < 0.0001; fig. 4b). The AUC for basophil activation was markedly reduced following exposure to tomato (p = 0.03; fig. 5a) and JCP (p < 0.0001; fig. 5b) extracts. We evaluated basophil activation by tomato extract in patients with and without oral allergy symptoms. No significant changes were observed in the AUC of basophil activation by tomato extract in the blood samples of patients with oral allergy symptoms from before
to after treatment (median values: 122.3 vs. 102.6, p = 0.63), but there were significant changes in the AUC of basophil activation by tomato fruit extract in the blood samples of patients without symptoms (median values: 91.4 vs. 53.2, p = 0.04). The percentage of CD203c-positive basophils in the presence of anti-IgE antibodies significantly decreased after SCIT (p = 0.001; fig. 6a) whereas the MFI of anti-FcεRI on basophils significantly increased (p = 0.0012; fig. 6b). We designated 1 patient with no basophil response to the positive control anti-IgE antibodies.

**Serum-Specific IgE and IgG4 Levels**

From before to after the treatment, the patients’ serum total IgE levels did not change significantly (median values: 806 vs. 893 IU/ml; p = 0.36). After SCIT, no significant changes were observed in specific IgE levels for tomato fruit (p = 0.11; fig. 7a) or JCP (p = 0.19; fig. 7b). Tomato fruit-specific IgG4 levels tended to increase, although not to levels that were significant (p = 0.32; fig. 7c), but the JCP-specific IgG4 levels increased significantly (p < 0.0001; fig. 7d). We also analyzed the ratio between specific IgE and specific IgG4 levels in patients’ serum. After SCIT, JCP-specific IgE/IgG4 levels had declined from before the treatment (median values: 169.3 vs. 43.2; p < 0.01), but tomato fruit-specific IgE/IgG4 levels had not changed (median values: 3.26 vs. 5.39; p = 0.34).

**Immunoblotting**

Several protein bands with a molecular weight range of 7–50 kDa reacted with the IgE from the patients (fig. 8). Among these bands, 39-kDa and 9-kDa protein bands exhibited nonspecific binding to goat IgG because they reacted with control serum IgE. The visualized density of the 46-kDa protein band became stronger after JCP-based SCIT.

**Discussion**

In this study, we investigated the effects of JCP-based SCIT on tomato allergy, focusing on the immunologic reactivity to tomato fruit after JCP-based SCIT. We sug-
**Fig. 6.** Percentages of CD203c-positive basophils in the presence of anti-IgE antibodies (a) and MFI of anti-FcεRI antibodies on basophils (b) before and after SCIT. Statistical comparisons were performed using the Wilcoxon matched-pairs signed-ranks test.

**Fig. 7.** The median values for tomato fruit-specific IgE levels (a), JCP-specific IgE levels (b), tomato fruit-specific IgG4 levels (c) and JCP-specific IgG4 levels (d) before and after SCIT. Statistical comparisons were performed using the Wilcoxon matched-pairs signed-ranks test.
gested that this therapy reduced basophil activation caused by tomato and JCP extracts.

Asero [4] showed that 84% of patients with pollen-induced apple allergy reported either a complete disappearance of or significant improvements in their symptoms during the follow-up period of 12–48 months after birch pollen immunotherapy whereas no control patients reported the resolution or amelioration of PFAS at their follow-up visits. In contrast, Hansen et al. [6] showed that sublingual immunotherapy and SCIT were similar to placebo with respect to improvements in apple-induced PFAS in 74 adults with birch pollen allergy.

The mechanisms underlying the effects of specific immunotherapy for type I allergies are poorly understood. However, the following immunologic mechanisms are plausible. Basophil activity is reduced during the early course of allergen-specific immunotherapy. This is followed by the generation of allergen-specific regulatory T cells and the subsequent suppression of allergen-specific Th1 and Th2 cells. An early increase and a very late decrease in specific IgE levels have also been observed. In particular, IgG4 levels show a relatively early increase in an immunotherapy dose-dependent manner [20].

BAT has been used to diagnose allergies to many allergens, including food, pollen, venom and drugs [21–25]. Ebo et al. [12] showed that the in vitro analysis of CD63 expression on basophils from individuals allergic to birch pollen with and without apple-induced PFAS was specific, sensitive and comparable to detecting relevant allergen-specific IgE levels. Recently, reduced basophil activation was demonstrated with food-specific immunotherapy, and the suppression of basophil activation in treated patients correlated with their treatment [15, 26].

BAT results in patients who underwent SCIT with JCP decreased 1 month after starting immunotherapy, and their suppression continued throughout a study period of 1 year [14]. In our study, the reduced basophil activation caused by tomato extract suggests that SCIT with JCP extract has a beneficial effect on JCP-associated tomato fruit allergy. Basophil activation caused by tomato extract in the blood samples of patients allergic to tomato fruit did not significantly change, whereas basophil activation caused by tomato extract in the blood samples of patients not allergic to tomato fruit did significantly change. We consider the following 2 factors to be the reasons for this result: the sample size may have been too small or the effect of JCP-based SCIT on tomato fruit allergy may have been weak.

In other reports on the effects of birch pollen-based SCIT in patients allergic to apple [4, 6, 8], serum birch pollen-specific IgE levels declined after SCIT, but the changes in serum apple-specific IgE levels were not consistent. We found insignificant increases in serum JCP-specific IgE levels. We considered the following 3 factors to be responsible for this result. First, the patients did not receive identical amounts of allergen extract [27]. Second, our follow-up period was shorter than those of previous studies. Third, we included patients whose tomato fruit-specific IgE levels were borderline-positive, so it might have been difficult for these levels to decline.

Bucher et al. [28] showed that both serum birch pollen-specific IgG4 levels and apple-specific IgG4 levels were significantly elevated in some patients. In our study, the trends we observed regarding changes in serum IgG4 levels specific to pollen and fruit were in agreement with those that they observed. Lalek et al. [29] showed that birch immunotherapy-induced IgG4 antibody levels were associated with reduced basophil activation by this allergen. Further studies are thus required to clarify the contribution of JCP-specific IgG4 antibodies to basophil activation by tomato extract.

The immunoblotting results showed that the tomato extracts in this study reacted with patients’ IgE, as in our previous study [19]. The visualized density of the 46-kDa protein band became stronger after JCP-based SCIT in only 2 of 5 participants. The protein has the same molecular weight as polygalacturonase, which we demonstrated exhibits cross-reactivity to JCP.

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Fig. 8. Immunoblot of tomato extract using patients’ serum before and after SCIT. A = After SCIT; B = before SCIT; MW = molecular weight.
We have already demonstrated cross-reactivity between the allergens in tomato fruit and JCP [2]. However, the association of cross-reactivity with this reduction in basophil activation remains uncertain. Apple is cross-reactive with birch pollen, but not with JCP. We therefore investigated basophil activation using apple extract in 7 apple-sensitized children with JCP-induced allergic rhinitis, using the same evaluation schedule used in this study. The apple fruit-specific basophil activation did not significantly change in these subjects (data not shown). SCIT with JCP extract reduces BAT results in the case of tomato extract, but not with apple. This result suggests that the cross-reactivity of allergens is associated with a change in BAT results.

Consistent with this study, some earlier studies also showed reduced basophil activation by anti-IgE antibodies after allergen-specific immunotherapy [15, 30]. This reduced basophil activation by anti-IgE antibodies and increased MFI for anti-FcεRI on basophils suggest that SCIT may induce basophil anergy. Anti-IgE stimulation is not a nonspecific activation. Although we attempted to evaluate activation by fMLP (N-formyl-methionyl-leucyl-phenylalanine), we were unable to determine the optimal concentration of fMLP in this study. The mechanisms underlying this reduced basophil activation to anti-IgE in patients with a pollen-associated fruit allergy after pollen SCIT require further study, including the evaluation of intracellular basophil reactions.

Our study had some limitations. First, only 5 patients had PFAS caused by tomato fruit; the others were only sensitized. Unfortunately, the number of patients allergic to tomato with concomitant JCP rhinitis is very small. Only 5 patients intended to undergo JCP-based SCIT. Therefore, in this study, we evaluated the immunologic efficacy of JCP-based SCIT in a group of participants that included patients sensitized only to tomato fruit. Fruit-sensitized patients have the potential to develop a fruit allergy [31]. We evaluated the efficacy of JCP-based SCIT to prevent the symptoms of tomato fruit allergy. Second, our follow-up period was relatively short (4 or 5 months), which did not allow for adequate assessments of other immunologic changes. Furthermore, only 1 patient with PFAS caused by tomato ate the fruit without developing symptoms; the other patients with PFAS refused to eat the fruit for fear of an allergic reaction. A longer-term follow-up and a double-blind food challenge will be necessary to confirm our findings. However, although our study was not controlled, PFAS does not normally improve without treatment [4, 28].

In conclusion, to the best of our knowledge, we showed for the first time that tomato fruit-specific basophil activation was reduced after JCP-based SCIT. This suggests that JCP-based SCIT may be effective for relieving and preventing the symptoms of PFAS in patients with JCP-induced allergic rhinitis.

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Disclosure Statement

The authors had no conflicts of interest related to this work.

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