



Immunological effects of sublingual immunotherapy with Japanese cedar pollen extract in patients with combined Japanese cedar and Japanese cypress pollinosis

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ABSTRACT

Sublingual immunotherapy (SLIT) with Japanese cedar (JCe) pollinosis was expected to be effective for Japanese cypress (JCy) pollinosis. However, only a half of JCy pollinosis patients clinically improved. Therefore, we examined the immunological effect of SLIT for JCy pollinosis. Peripheral blood mononuclear cells (PBMCs) from patients with JCe and JCy pollinosis who did and did not receive SLIT were incubated with Cry j 1, Cha o 1 and Cha o 3 antigens. Basophil activation test (BAT) were performed. Production of IL-5 and IL-17 induced by antigens was inhibited in the SLIT group. Cry j 1-specific production of IL-10 was increased, and serum Cry j 1-specific IgE and -IgG4 were elevated. However, Cha o 1- or Cha o 3-specific production of IL-10 and specific IgG4 was not increased. Antigens-specific BAT did not decrease after SLIT. New SLIT with JCe and JCy is needed for patients with combined JCe and JCy pollinosis.

1. Introduction

Seasonal allergic rhinitis (AR) caused by Japanese cedar (JCe) pollen is the most common allergic disease in Japan. More than one-third of the population has JCe pollinosis, and its prevalence has significantly increased in the last two decades. Large amounts of JCe pollen are dispersed over long distances (> 100 km) in the allergy season from February to April. Furthermore, pollen from Japanese cypress (JCy), which also causes seasonal AR, are dispersed in April and May, just after dispersal of JCe pollen, and approximately 70% of patients with JCe pollinosis also have JCy pollinosis [1]. Therefore, many patients with combined JCe and JCy pollinosis experience severe symptoms for as long as 4 months from February to May.

Allergen specific immunotherapy (AIT) is the only curative treatment for AR. Sublingual immunotherapy (SLIT) with standardized JCe

pollen extract has recently become available for treatment of JCe pollinosis in Japan, and has been supported by Japanese health insurance since 2014. Cry j 1 and Cry j 2 are major allergens in JCe pollen that have strong amino acid sequence homology with the major allergens of JCy pollen, Cha o 1 and Cha o 2, respectively [2,3]. This homology causes cross-reactivity with some T-cell epitopes and allergen-specific IgE [4–7], which suggests that SLIT with standardized JCe pollen extract may be effective for both JCe pollinosis and JCy pollinosis. However, only a half of patients with combined JCe and JCy pollinosis have improved symptoms after SLIT in JCy pollen season, whereas > 80% show improved symptoms in JCe pollen season [8,9].

In 2016, a new major allergen of Japanese cypress pollen, Cha o3, was discovered, and the response above suggests that SLIT with JCe pollen extract might not control Cha o3-specific T_H2 responses [10,11]. The mechanism of action of SLIT is not fully understood, but it has been

Abbreviations: AIT, allergen specific immunotherapy; AR, allergic rhinitis; BAT, basophil activation test; ELISA, enzyme-linked immunosorbent assay; FOXP3, forkhead box P3; IFN, Interferon; IL, Interleukin; iTreg, inducible regulatory T cells; JAU, Japanese allergy units; JCe, Japanese cedar; JCy, Japanese cypress; nTregs, natural regulatory T cells; OD, optical density; PBMCs, peripheral blood mononuclear cells; SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy; TGF, transforming growth factor; TNSMS, total nasal symptom and medication scores; Tregs, regulatory T cells; VAS, visual analog scale.

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suggested that the T_H1/T_H2 ratio is increased, IL-10 producing regulatory T cells (Tregs) are induced, and allergen-specific IgG4 is produced in SLIT. This study was performed to examine the immunological effects of SLIT with JCe pollen extract in patients with combined JCe and JCy pollinosis by measuring (1) Cry j 1-, Cha o 1- and Cha o 3-induced production of IL-5, IL-10, IL-17 and IFN- γ from PBMCs; (2) serum levels of Cry j 1-, Cha o 1- and Cha o 3-specific IgE and IgG4 in patients with or without SLIT; and (3) Cry j 1-, Cha o 1- and Cha o 3-induced basophil activation to study the functional allergen-specific IgE. (4) Antigen-specific basophil activation test was performed after SLIT.

2. Methods

2.1. Patients

This prospective study included 20 healthy controls, 25 patients with combined JCe and JCy pollinosis who were not treated with SLIT (non-SLIT group), and 82 patients with combined pollinosis treated with SLIT (SLIT group) at the Department of Otorhinolaryngology, Shiga University Hospital, Otsu, or Yuta Clinic, Tsu, Japan (Table 1). Seasonal AR was defined as the presence of typical nasal symptoms during pollen season, and a serum JCe-specific IgE and JCy-specific IgE ≥ 0.70 UA/mL (i.e., CAP-RAST class ≥ 2). There was no significant difference in serum CAP-RAST class between the non-SLIT and SLIT groups. The exclusion criteria were the presence or history of asthma or asthma-like symptoms. No patient had been treated with systemic corticosteroids for at least 4 weeks before sample collection. The healthy controls had no history of atopic disorders and a negative serum CAP-RAST class for JCe and JCy pollens. All patients gave written informed consent before sample collection. SLIT with standardized JCe pollen extract (Torii Pharmaceutical Co. Ltd., Tokyo, Japan) was administered over a period of 1 to 2 years. The SLIT protocol started with a 2-week dose escalation phase, followed by a maintenance phase of 1.0 mL of standardized JCe pollen extract (2000 Japanese allergy units {JAU}) containing 1.46 to 4.2 μ g/mL of Cry j 1 administered daily [12,13]. This study is cross-sectional study. Treatment policies were determined based on general medical care and are independent of clinical trials with interventional approach. This study conforms to the standards of the Declaration of Helsinki and was approved by the institutional review board of Shiga University of Medical Science, Otsu, Japan (approval number: 29–211).

2.2. Total nasal symptom and medication scores (TNSMS) and response rate of SLIT

Total nasal symptom and medication scores (TNSMS: range 0–18) and visual analog scales (VAS: 0–100) for nasal and eye symptoms were evaluated in the peak JCe pollen and JCy pollen seasons in 2016 (Table 2). Based on the Japanese guidelines for AR, symptom scores

Table 2

VAS, TNSMS and response rate after SLIT in patients with combined Japanese cedar and cypress pollinosis.

	Japanese cedar pollen season	Japanese cypress pollen season
VAS score	4.19 $\pm$ 0.28	4.55 $\pm$ 0.33
TNSMS	23.96 $\pm$ 2.40	25.3 $\pm$ 2.76
Effective Response rate	79.5%	37.3%

were evaluated for sneeze, rhinorrhea and nasal congestion on 5-point scales (0 = none, 1 = mild, 2 = moderate, 3 = severe, 4 = very severe). Ocular symptoms (itchiness and watery eyes) were scored on a 4-point scale (0 = none, 1 = mild, 2 = moderate, 3 = severe). Patients were allowed to use drugs such as antihistamines. Use of any medication was scored as 0 (not used) or 3 (used). We evaluated response rate of SLIT for combined Japanese cedar and Japanese cypress pollinosis ($n = 82$) with questionnaire results. The response rate of SLIT was evaluated at the end of the pollen season by comparing the 6-grade severity scores (greatly improvement, improvement, slightly improvement, no change, slightly worsening, or worsening). Patients who were described as greatly improvement, and improvement on the questionnaire were considered effective responders (Table 2).

2.3. Cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated from 20 mL of heparinized venous blood by density gradient centrifugation using Ficoll-Paque Premium (GE Health Care, Uppsala, Sweden). PBMCs (2×10^6 cells/mL) were cultured in RPMI 1640 medium (Wako Pure Chemical Industries, Osaka, Japan) containing 10% autologous serum, and were incubated in 96-well round bottom plates (Falcon; Corning Inc., Corning, NY, USA) with 10 μ g/mL of Cry j 1 (Hayashibara Co., Ltd., Okayama, Japan), Cha o 1 or Cha o 3 (provided by T Osada) at 37 °C in 5% CO₂ for 5 days. Then, cell-free supernatants were analyzed for IL-5, IL-17, IL-10 and IFN- γ (R&D Systems, Minneapolis, MN, USA) using commercial enzyme-linked immunosorbent assay (ELISA) kits.

2.4. Allergen-specific IgE and IgG4

Serum concentrations of allergen-specific IgE and IgG4 were measured by established ELISA procedures [10,14]. Briefly, allergen solution (IgE: 20 μ g/mL, IgG4: 100 ng/mL) was prepared at 100 mM in sodium bicarbonate and incubated in a Maxisorp 96-well plate (Thermo Fisher Scientific, Waltham, MA, USA) overnight at 4 °C to coat wells with allergens. After washing 5 times with wash buffer (1% BSA in PBS/0.05% Tween 20), blocking buffer (1% BSA in PBS) was added to each well and incubated for 1 h at room temperature. After plate washing, 5-fold (IgE) or 2-fold (IgG4) diluted serum samples or control serum was added and incubated for 2 h at room temperature. The plate was

Table 1

Characteristics of the subjects.

Variable	Healthy controls ($n = 20$)	Non-SLIT ($n = 25$)	SLIT ($n = 82$)
Age (years)	36 $\pm$ 3	36 $\pm$ 3	43 $\pm$ 2 [*]
Sex (male/female)	9 / 11	17 / 8	47/35
Japanese cedar pollen sIgE (class)	0	3.50 $\pm$ 0.23	3.85 $\pm$ 0.12
Japanese cypress pollen sIgE (class)	0	2.28 $\pm$ 0.18	2.33 $\pm$ 0.09
Japanese cedar pollen sIgE (UA/mL)	0	22.08 $\pm$ 4.33	33.45 $\pm$ 3.5
Japanese cypress pollen sIgE (UA/mL)	0	3.2 $\pm$ 0.72	4.58 $\pm$ 0.74

non-SLIT: patients with pollinosis who did not receive sublingual immunotherapy (SLIT).

SLIT: patients with pollinosis who were treated with SLIT.

sIgE: specific IgE.

^{*} $P < .05$.

washed 5 times, and 7000-fold diluted horseradish peroxidase-labeled goat antihuman IgE antibody (Novus Biologicals, Centennial, CO, USA) or 500 ng/mL biotinylated monoclonal anti-IgG4 antibody (BD Pharmingen) and 40-fold-diluted streptavidin-HRP (R&D Systems) were added. Substrate solution was prepared with a TMB Substrate Reagent Set (BD Biosciences, San Jose, CA, USA), and an equal volume of 2 M H₂SO₄ was added to each well to stop the reaction. Optical density (OD) at 450 nm was measured using a microplate reader (Bio-Rad Laboratories, Irvine, CA, USA).

2.5. Basophil activation test (BAT)

Heparinized whole blood was analyzed using an Allergen kit (Beckman Coulter, Indianapolis, IN, USA) [10]. Allergen solutions (1 ng/mL) and 20 µL of an antibody cocktail of anti-human CD3-phycoerythrin (PE) cyanin 7 (PC7), anti-human CRTH2-fluorescein isothiocyanate (FITC), and anti-human CD203c-PE antibodies were added to 100 µL of heparinized whole blood. The samples were incubated for 15 min at 37 °C with avoidance of light. Then, 100 µL of stop solution and 2 mL of "Fix-and-Lyse mixture" were added to each sample and incubated for 10 min while avoiding light. Cells were collected by centrifugation and analyzed by flow cytometry (BD FACSCanto, BD Biosciences).

2.6. Statistical analysis

Data from two independent groups were compared by Mann-Whitney *U* test, and those from three independent groups were compared by Kruskal-Wallis test. Correlations were assessed by Spearman rank correlation analysis. *P* < .05 was considered significant. All analyses were performed using IBM SPSS Statistics (v. 22.0, IBM Inc., New York, NY, USA).

3. Results

3.1. Antigen-induced production of IL-5, IL-17, IFN-γ and IL-10 from PBMCs

Effects of SLIT on antigen-induced production of IL-5, IL-17, IFN-γ and IL-10 from PBMCs were examined using blood samples collected from patients out of pollen seasons. Cry j 1, Cha o 1 and Cha o 3 significantly induced IL-5 production from PBMCs in the non-SLIT group compared with healthy controls, and this effect was significantly inhibited in PBMCs of the SLIT group (Fig. 1A). Cry j 1, Cha o 1 and Cha o 3-induced IL-17 production from PBMCs of healthy controls and the non-SLIT group was significantly inhibited in the SLIT group (Fig. 1B). Cry j 1, Cha o 1 and Cha o 3-induced IFN-γ production from PBMCs of healthy controls was also attenuated in the SLIT group (Fig. 1C). Cry j 1-specific IL-10 production from PBMCs in the SLIT group was significantly higher than that in the non-SLIT group, whereas Cha o 1- or Cha o 3-induced IL-10 production from PBMCs in the non-SLIT group was inhibited in the SLIT group (Fig. 1D).

3.2. Serum concentrations of antigen-specific IgE and IgG4

Serum Cry j 1-, Cha o 1-, and Cha o 3-specific IgE were elevated in the non-SLIT group, but only Cry j 1-specific IgE was significantly increased in the SLIT group compared with the non-SLIT group (Fig. 2A). Cry j 1-specific IgG4 was only increased in the SLIT group (Fig. 2B). SLIT did not affect the serum levels of Cha o 1- or Cha o 3-specific IgE and IgG4.

3.3. Correlations of severity of clinical symptoms with antigen-induced cytokine production and serum concentrations of antigen-specific IgG4

Cry j 1-induced production of IL-10 from PBMCs relative to that of

IL-5 (IL-10/IL-5 ratio) in the SLIT group was negatively correlated with clinical symptoms evaluated by VAS scores in the peak cedar pollen season. However, the Cha o 1- or Cha o 3-induced IL-10/IL-5 ratio was not correlated with VAS scores in the peak cypress pollen season (Fig. 3A, B). In the peak cedar pollen season, the Cry j 1-induced IL-10/IL-5 ratio was not correlated with TNSMS (data not shown), whereas the serum level of Cry j 1-specific IgG4 in the SLIT group was negatively correlated with TNSMS (Fig. 4), but not correlated with VAS scores (data not shown).

3.4. Basophil activity test (BAT)

Cry j 1 significantly induced basophil activation in the non-SLIT group compared with the healthy controls, and this change was suppressed in the SLIT group, although without significance (Fig. 5). This suggests that Cry j 1 is important in the pathogenic mechanism of JCe pollinosis, but a clinical effect of SLIT was not supported by the BAT results. Cha o 1- or Cha o 3- induced basophil activation did not differ significantly between the non-SLIT and SLIT groups.

4. Discussion

In this study, Cry j 1, Cha o 1 and Cha o 3 significantly induced IL-5 production from PBMCs of patients with combined JCe and JCy pollinosis, and were significantly inhibited after SLIT with standardized JCe pollen extract. Interestingly, only Cry j 1 significantly induced IL-10 production from PBMCs after SLIT, and serum Cry j 1-specific IgE and IgG4 were elevated. Cry j 1-induced IL-10/IL-5 ratios and serum Cry j 1-specific IgG4 levels were negatively correlated with the severity of clinical symptoms evaluated by VAS scores and TNSMS, respectively, in peak JCe pollen season. These results indicate that SLIT with JCe pollen extract non-specifically suppresses Cry j 1-, Cha o 1- or Cha o 3-induced IL-5 production from PBMCs, and induces antigen (Cry j 1)-specific IL-10 production from PBMCs and antigen (Cry j 1)-specific IgE and IgG4 production in serum. This is the first report showing the immunological effects induced by JCe and JCy antigens after SLIT with JCe pollen extract.

An influence of SLIT on T_H cells has been reported in patients with seasonal pollen AR, and the efficacy of SLIT is associated with a decrease in antigen-specific T_H2 cytokine-producing T cells [13]. These results are supported by the decrease of T_H2 cytokine levels in nasal lining fluid after nasal challenge [15]. SLIT with JCe pollen extract significantly inhibits JCe pollen-induced production and mRNA expression of IL-4, IL-5 and IL-13 in PBMCs of patients with JCe pollinosis [16,17]. JCe pollen-specific IL-4- and IL-5-producing T cells are increased in these patients during pollen season, and are significantly inhibited after SLIT [13,17,18]. These results are consistent with the present study, in which SLIT with JCe pollen extract inhibited Cry j 1-induced IL-5 production from PBMCs. In addition, we found that Cha o 1- and Cha o 3-induced IL-5 production was significantly inhibited after SLIT with JCe pollen extract, and that Cry j 1, Cha o 1 and Cha o 3-induced IL-17 and IFN-γ production was also inhibited, suggesting that SLIT induces non-specific suppression of T_H cells.

Immune tolerance after SLIT is associated with induction of regulatory T cells (Tregs) [15]. Two major subtypes of Tregs have been identified: natural (nTregs) and inducible (iTregs). nTregs express the transcription factor forkhead box P3 (FOXP3) and IL-25 receptor (CD25), and iTregs include additional subtypes (T_R1 and T_R3) that produce suppressive cytokines such as IL-10 and TGF-β, respectively. Subcutaneous immunotherapy (SCIT) and SLIT with grass pollen are associated with local increases of FOXP3⁺CD25⁺ T cells in nasal mucosa and FOXP3⁺ T cells in sublingual mucosa [19,20]. Interestingly, our results revealed that JCy allergen component, Cha o 1 and Cha o 3 induced-cytokine production (IL-5, IL-17 and IFN-γ) were also inhibited after SLIT for JCe pollinosis. We speculated that increased natural regulatory T cells (nTreg) which exert their suppressive capacity in a

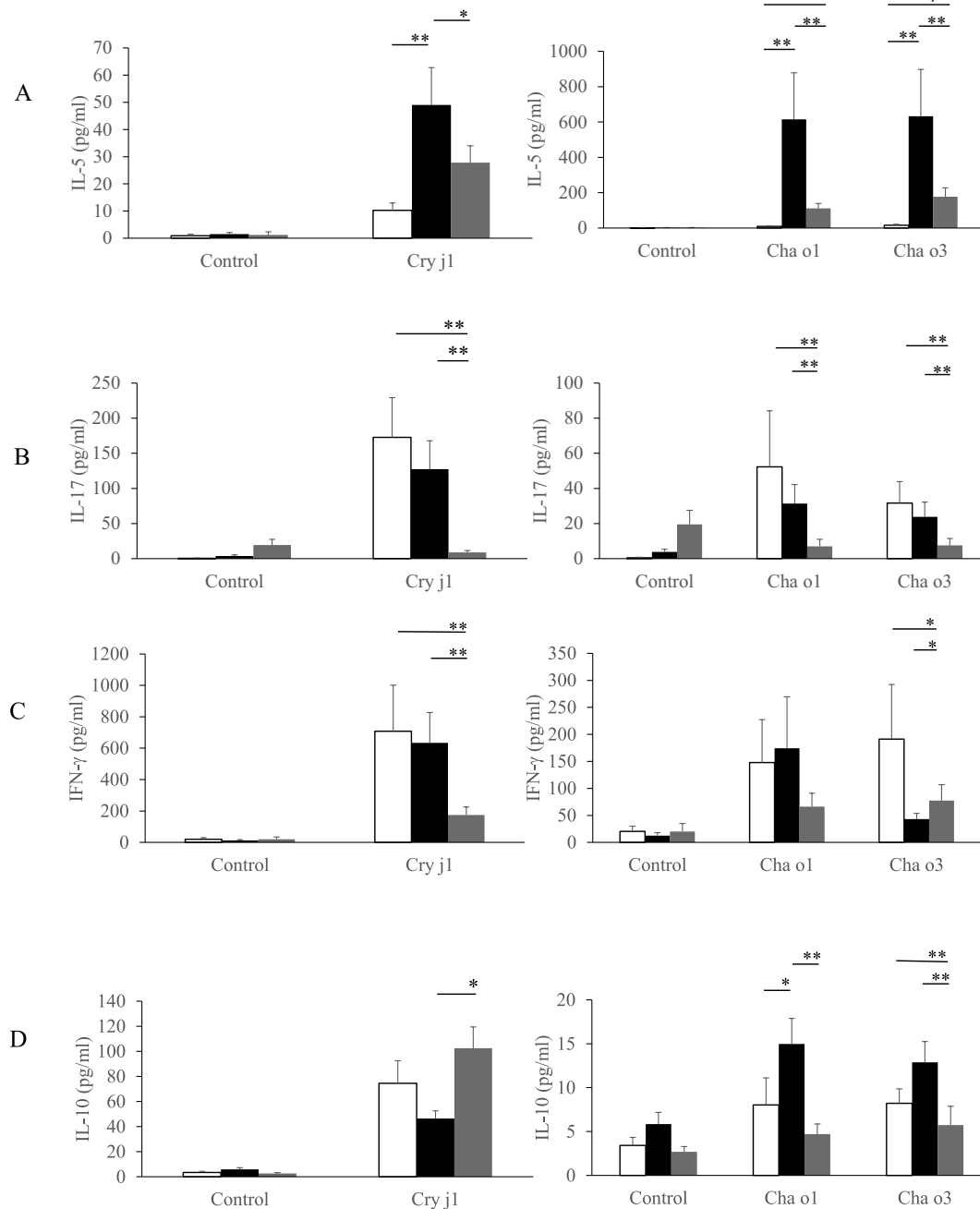


Fig. 1. Antigen-induced production of IL-5, IL-17, IFN- γ and IL-10 from cultured PBMCs. PBMCs (2×10^6 cells/mL) were incubated with 10 μ g/mL of Cry j 1, Cha o 1 or Cha o 3 for 5 days. Antigen-induced production of IL-5 (A), IL-17 (B), IFN- γ (C) and IL-10 (D) was assayed by ELISA. White bars: healthy controls ($n = 20$); black bars: non-SLIT group ($n = 20$); gray bars: SLIT group ($n = 56$). * $P < .05$, ** $P < .01$.

directly cell-cell contact-dependent manner may be involved in these cytokine suppressions.

AIT-induced IL-10, IL-35 and TGF- β -producing iTregs suppress antigen-induced T cell proliferative responses and T_H2 cytokine release [15,21,22]. Increased proportions of circulating IL-10- and IL-35-producing Tregs and increased IL-10- and TGF- β -producing T cells in nasal mucosa are present after SCIT or SLIT with grass pollen [19,23–25]. SLIT with JCe pollen extract significantly increased the proportions of circulating JCe pollen-specific IL-10-producing Tregs and IL-10-producing B cells (Bregs) [26,27], and these cells are associated with the clinical efficacy of this SLIT [18,28]. IL-10 enhances survival, proliferation, differentiation and isotype switching of B cells, resulting in IgG4 production [21]. Moreover, Bregs support the differentiation of

Tregs, induce the inhibition of proinflammatory response and augment IgG4 production [29]. Antigen-specific IgG4 works as a blocking antibody and is involved in the mechanism of SLIT, and serum Cry j 1-specific IgG4 is elevated after SLIT with JCe pollen extract in association with the increase in IL-10-expressing T cells or B cells [14,18,28].

In this study, Cry j 1 significantly induced IL-10 production from PBMCs after SLIT, and the serum level of Cry j 1-specific IgG4 was elevated. These changes were associated with the clinical efficacy of SLIT based on VAS scores and TNSMS in peak JCe pollen season. The amino acid sequences of Cry j 1 and Cha o 1 are 80% identical, and cross-allergenicity has been shown based on serum IgE from JCe pollenosis patients being cross-reactive with Cry j 1 and Cha o 1 [5–7], and by T cell epitopes common to Cry j 1 and Cha o 1 [4]. However, Cha o 1

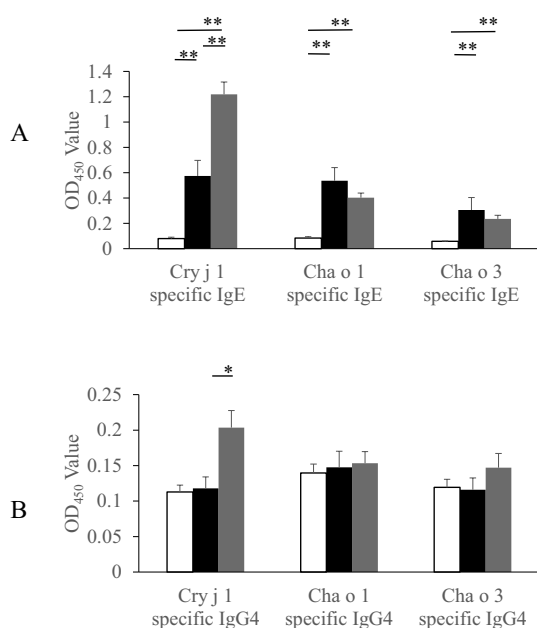


Fig. 2. Serum concentrations of antigen-specific IgE and IgG4. Serum levels of Cry j 1-, Cha o 1- and Cha o 3-specific IgE (A) and IgG4 (B) were assayed by ELISA. The relative levels of antigen-specific IgE and IgG4 are expressed as optical density (OD) values. White bars: healthy controls ($n = 20$); black bars: non-SLIT group ($n = 25$); gray bars: SLIT group ($n = 82$). * $P < .05$, ** $P < .01$.

had no effect on IL-10 production from PBMCs, and Cha o 1-specific IgG4 was not detected in this study. These results suggest that antigen-specific induction of Tregs or Bregs was only induced by cedar allergen (Cry j 1) after SLIT with JCe pollen extract, despite the cross-allergenicity between cedar (Cry j 1) and cypress (Cha o 1) pollens.

Cha o 3 is a newly discovered major allergen of JCy pollen [10], and a new Cha o 3 homolog, Cry j 4, was found in JCe pollen in 2018. The amino acid sequences of the two proteins are 84% identical, and cross-allergenicity between Cha o 3 and Cry j 4 has been shown at the T cell and IgE levels [11]. However, SLIT with JCe pollen extract may not

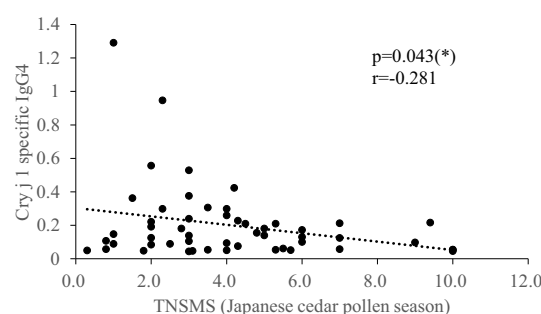


Fig. 4. Correlation between severity of clinical symptoms and serum levels of Cry j 1-specific IgG4 after SLIT. Total nasal symptom and medication scores (TNSMS) in peak cedar pollen season were correlated with the serum concentration of Cry j 1-specific IgG4 after SLIT. * $P < .05$.

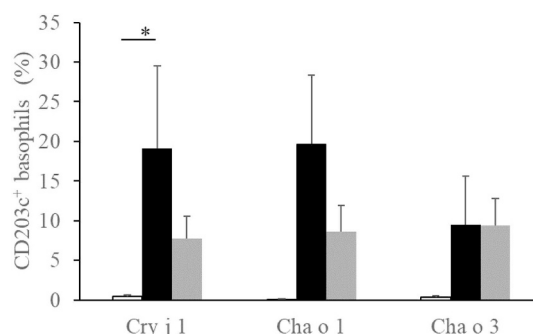


Fig. 5. Basophil activation test (BAT). The data show basophil activation induced by Cry j 1, Cha o 1 or Cha o 3. White bars: healthy controls ($n = 9$); black bars: non-SLIT group ($n = 8$); gray bars: SLIT group ($n = 10$). * $P < .05$.

induce adequate immune tolerance to Cha o 3 because of the low amount of Cry j 4 in JCe pollen. As a result, Cha o 3 has no effect on IL-10 production, and Cha o 3-specific IgG4 was not detected in this study.

Basophil activation has been studied by monitoring expression of a surface activation marker, CD203c. Basophils are effector cells that are critically involved in the pathophysiology of AR, and the BAT is a highly sensitive test for functional IgE sensitization compared with the

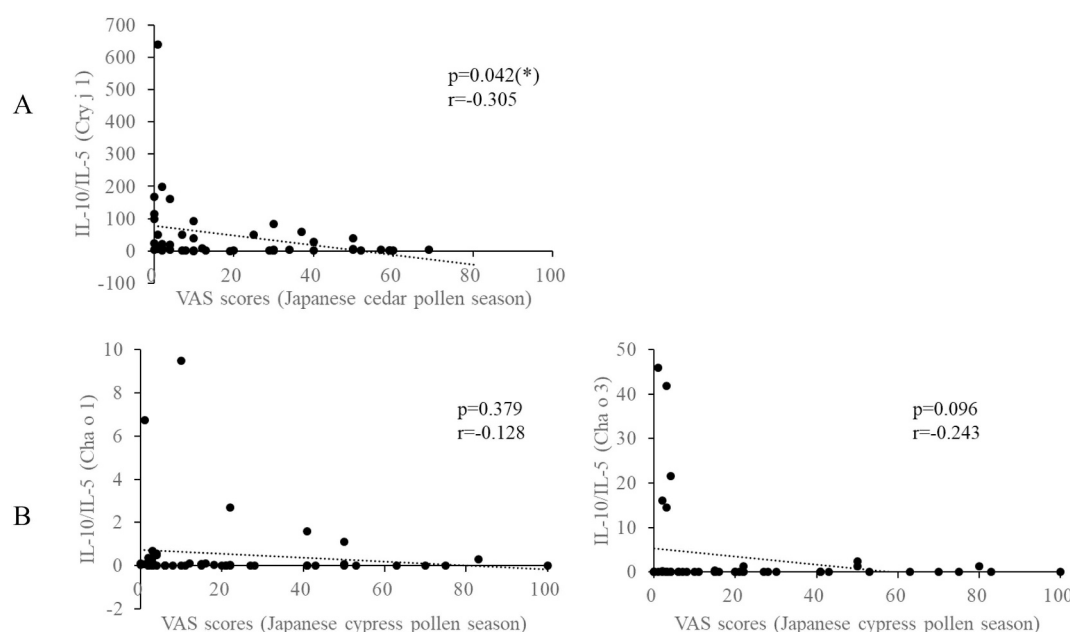


Fig. 3. Correlations between severity of clinical symptoms and antigen-induced cytokine production after SLIT. Correlations of visual analog scales (VAS) scores for clinical symptoms in peak cedar pollen season with (A) Cry j 1-, (B) Cha o 1-, and (C) Cha o 3-induced IL-10/IL-5 ratios from PBMCs in the SLIT group. * $P < .05$.

serum level of antigen-specific IgE. Basophil activation is thought to be reduced by induction of antigen-specific IgG4 (blocking antibody), and a BAT has been used to evaluate the efficacy of AIT to bee venom, olive, house dust mite and grass pollen allergens [30–34]. However, the results are unclear, and basophil activation has been reported to not be associated with early efficacy of SLIT with grass pollen [33]. In the present study, Cry j 1, Cha o 1 and Cha o 3 induced basophil activation in patients with combined JCe and JCy pollinosis, but the clinical efficacy of SLIT was not supported by the BAT results. It is possible that the reactivity of circulating basophils does not reflect those in nasal mucosa, and that a longer treatment duration is required for changes in the BAT.

In conclusion, we showed that SLIT with JCe pollen extract induces suppression of Cry j 1, Cha o 1 and Cha o 3-derived IL-5 production, Cry j 1-specific IL-10 production, and Cry j 1-specific IgG4 production in patients with combined JCe and JCy pollinosis. However, SLIT did not induce JCy antigen (Cha o 1 and Cha o 3)-specific IL-10 production, and JCy antigen-specific IgG4 was not detected. The Cry j 1-induced IL-10/IL-5 ratio, and the serum level of Cry j 1-specific IgG4 were associated with the efficacy of SLIT only in the JCe pollen season. Therefore, there is a need for new SLIT with JCe and JCy pollen extract for patients with combined JCe and JCy pollinosis.

Author contributions

HKikuoka, HKouzaki, TO, MO, AY and TS designed the study and HKikuoka wrote the manuscript. HA, SY, IT, SS, YO and HM contributed to sample collection. All authors read and approved the final manuscript.

Declaration of Competing Interest

TO is employees of Taiho Pharmaceutical Co., LTD. AY has received fee for speaking from Torii Co., LTD in this work. TS receives scholarship donations from Shionogi & Co., LTD. But, none of the authors has any potential financial conflict of interest related to this manuscript.

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