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# Local expression of interleukin-17a is correlated with nasal eosinophilia and clinical severity in allergic rhinitis

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## ABSTRACT

*Interleukin (IL)-17A is a major cytokine produced by Th17 cells, which are associated with chronic inflammations. The local expression of IL-17A in allergic rhinitis (AR) remains to be characterized. We sought to determine the role of IL-17A expression in human inferior turbinate mucosa in the pathophysiology of AR. Inferior turbinate mucosa was sampled from medical treatment–resistant, surgery-required patients with perennial AR (PAR, n = 21), nonallergic rhinitis with eosinophilia syndrome (NARES, n = 7), and nonallergic hypertrophic rhinitis (HR, n = 13). IL-17A expression was determined with immunohistochemical staining. The mean number of IL-17A$^+$ cells and eosinophils per field were counted. Total serum immunoglobulin E (IgE) levels, blood eosinophil count, and forced expiratory volume in 1 second (FEV$_1$)/forced vital capacity (FVC) ratio were also examined in each patient. IL-17A was primarily expressed in infiltrating inflammatory cells. The number of IL-17A$^+$ cells in nasal mucosa was significantly higher in the PAR group compared with HR (p = 0.002) and NARES (p = 0.021) groups. There was a significant and positive correlation between the number of IL-17A$^+$ cells and total nasal symptom score (rho = 0.403; p = 0.011), especially sneezing score (rho = 0.471; p = 0.003). The number of IL-17A$^+$ cells was significantly and positively correlated with the degree of eosinophil infiltration (rho = 0.623; p < 0.001), but not with total serum IgE levels (rho = 0.284; p = 0.098), blood eosinophil counts (rho = 0.302; p = 0.056), or FEV$_1$/FVC ratio (rho = 0.092; p = 0.569). The present study provides evidence that IL-17A expression in the nasal mucosa is associated with the pathophysiology of AR, including disease severity and nasal eosinophilia.*

Allergic rhinitis (AR) is the inflammation of nasal mucosa, and allergen-specific CD4$^+$ Th2 cells, which produce interleukin (IL)-4, IL-5, IL-13, and IL-31, are believed to play a central role in its pathogenesis.[1,2] Other CD4$^+$ T-cell subsets, including Th1, Tr1, and Treg cells, can regulate Th2 responses and inflammation of AR.[3–5] Recently, Th17 cells were characterized as a distinct lineage of CD4$^+$ T cells and were found to be associated with autoimmune diseases and involved in the protection against microbial infections and chronic inflammation.[6,7]

IL-17A is a proinflammatory cytokine synthesized by Th17 cells.[6] IL-17A acts on a broad range of respiratory cells to induce the expression of cytokines, chemo-kines, matrix metalloproteinase proteins, and mucus proteins.[8,9] For example, we have recently reported that IL-17A expression is associated with eosinophilic inflammation in chronic rhinosinusitis (CRS) both *in vivo* and *ex vivo*.[10]

Research on the role of IL-17A in the pathogenesis of AR has accumulated.[11–19] Ciprandi *et al.* showed that serum concentrations of IL-17A are significantly elevated in patients with AR compared with healthy controls, and a significant positive relationship between serum IL-17A levels and symptom severity was observed.[11,12] They also found that peripheral blood mononuclear cells from AR patients have higher frequencies of IL-17A producing T cells and CD161$^+$ circulating T cells compared with those from normal subjects.[13,16] Neiminen *et al.* indicated that specific allergen-induced IL-17A mRNA expression in peripheral blood mononuclear cells of pediatric patients with AR was significantly and positively correlated with the symptom–medication score.[14] Additionally, Xu *et al.* showed that IL-17A levels in nasal lavages of patients with AR were significantly higher compared with those of controls. They further indicated that IL-17A enhanced CCL-20 and IL-8 expression in human nasal epithelial cells.[15] More recently, Baumann *et al.* found a significant increase of IL-17A in the nasal lavages of patients with seasonal AR after nasal allergen challenge.[20] On the other hand,

Table 1  Subject characteristics

|  | HR | NARES | PAR |
|---|---|---|---|
| No. of patients | 13 | 7 | 21 |
| Sex (male/female) | 11/2 | 6/1 | 16/5 |
| Age (yr) | 37 (24–57) | 44 (21–58) | 31 (20–57) |
| CAP titer to *Dermatophagoides farinae* (UA/mL) | 0 (0–0) | 0 (0–0) | 42.2 (0.55 to >100) |
| Total IgE (IU/mL) | 77 (5–182) | 149 (36–280) | 1139 (24–9831) |
| Blood eosinophil ($\mu L^{-1}$) | 171 (68–610) | 156 (34–305) | 409 (37–1754) |
| $FEV_1$/FVC ratio | 82 (70–100) | 82 (72–92) | 86 (67–100) |
| Patients with asthma | 0 | 0 | 3 |

HR = *hypertrophic rhinitis*; NARES = *nonallergic rhinitis with eosinophilic syndrome*; PAR = *perennial allergic rhinitis*; IgE = *immunoglobulin E*; $FEV_1$ = *forced expiratory volume 1 s*; FVC = *forced vital capacity*.

Groger *et al.* showed that elevated levels of IL-17A in nasal secretions were found in patients with nonallergic rhinitis with eosinophilia syndrome (NARES) compared with healthy controls as well as AR.[17] Mouse models have shown that IL-17A contributes to the development and regulation of AR.[18,19,21] However, local IL-17A expression in AR remains to be characterized.

In the present study, we sought to determine the expression of IL-17A in human inferior turbinate mucosa and compared the expression between patients with AR and nonallergic rhinitis. Furthermore, we analyzed the correlations between IL-17A expression in nasal mucosa and various pathophysiological parameters. We believe that the results presented here may provide insight into the role of IL-17A and Th17 in the pathophysiology of AR.

## MATERIALS AND METHODS

### Patients

Twenty-one Japanese patients with perennial AR (PAR), 7 patients with NARES, and 13 patients with nonallergic hypertrophic rhinitis (HR) were enrolled in the study. All of them presented with persistent nasal obstruction, were resistant to medical treatment, and underwent endoscopic nasal surgery (inferior turbinectomy with or without septoplasty). PAR and NARES were defined based on the Practical Guideline for Management of Allergic Rhinitis in Japan.[22] Patients having CRS were excluded. All of the PAR patients were sensitized with *Dermatophagoides farinae*, as confirmed by the presence of specific immunoglobulin E (IgE) antibodies (range, 0.55 to >100 UA/mL; mean, 42.22 ± 40.54 UA/mL), which were detected *via* an ImmunoCAP kit (Phadia AB, Uppsala, Sweden). Conversely, HR patients did not show nasal eosinophilia or sensitization to airborne allergens related to the symptoms. Three patients with PAR were asthmatic, and no patients had aspirin sensitivity. None of the participants received

systemic steroid treatment for a period of at least 8 weeks before surgery, and none received pharmacotherapy for rhinitis, such as intranasal steroids, for a period of at least 3 weeks before surgery. Patients treated with allergen-specific immunotherapy were excluded. Before surgery, we examined the total serum IgE levels, blood eosinophil count, and forced expiratory volume in 1 second/forced vital capacity ratio in each patient. The severity of nasal symptoms was graded according to the criteria outlined by Okuda *et al.*, in which three nasal symptoms (*i.e.*, sneezing, rhinorrhea, and nasal congestion) were rated on a 4-point scale from 0 to 3 (0 = no symptoms, 1 = minimal, well-tolerated symptoms, 2 = bothersome but tolerated symptoms, 3 = severe and hard to tolerate symptoms).[23] The clinical characteristics of the patients are presented in Table 1. All patients provided informed consent before their participation, and the study was approved by the Human Research Committee of the Okayama University Graduate School of Medicine and Dentistry.

### Immunohistochemistry

During surgery, the mucosa of the inferior turbinate was sampled from all of the patients. We performed immunohistochemical staining for IL-17A, according to a previously described protocol.[22] Briefly, 4-$\mu$m sections were collected from paraffin-embedded tissue blocks, deparaffinized, and rehydrated. The sections were incubated with trypsin for antigen retrieval and primary antibody, including 1:50 diluted rabbit anti-human IL-17A polyclonal antibody (H-132; Santa Cruz Biotechnology, Santa Cruz, CA) or control serum (Universal Negative Control; Dako Japan, Tokyo, Japan), at 4°C overnight. A Histofine MAX-PO(R) (Nichirei Bioscience, Tokyo, Japan) with diaminobenzidine substrate was used according to the manufacturer's instructions. The sections were then nuclear stained with hematoxylin and examined under a light microscope.
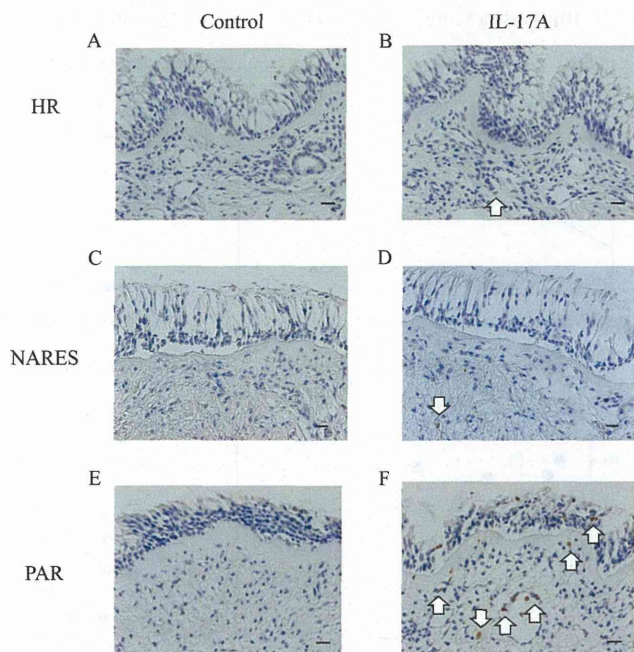
**Figure 1.** *Immunohistochemical staining of interleukin (IL)-17A in nasal mucosa from a patient with (A and B) nonallergic hypertrophic rhinitis (HR), (C and D) nonallergic rhinitis with eosinophilia syndrome (NARES), and (E and F) perennial allergic rhinitis (PAR). Sections were reacted with rabbit polyclonal antibody against (B, D, and F) IL-17A (A, C, and E) or control after which they were stained using a Histofine MAX-PO (Nichirei Bioscience, Tokyo, Japan) with a diaminobenzidine substrate. Arrows indicate IL-17+ cells (scale bar = 20 μm).*

Positive-stained cells were counted in five fields at high power (10 × 40), where the highest cellular infiltration was observed. The mean number of positive cells was then determined. Additionally, sections were stained with hematoxylin and eosin, and the number of eosinophils that had infiltrated into the nasal mucosa was counted in the same manner.

### Statistical Analysis

Values are presented as median values. A nonparametric Mann-Whitney $U$ test was used for comparing data between groups. A correlation analysis was performed using a nonparametric Spearman's correlation coefficient by rank. A value of $p < 0.05$ was considered statistically significant. Statistical analyses were performed using StatView software (Version 4.5; Abacus Concepts, Berkeley, CA).

### RESULTS

#### Local Expression of IL-17A in Nasal Mucosa

We immunohistochemically examined the expression and distribution of IL-17A in the nasal mucosa of inferior turbinate. IL-17A protein was primarily expressed in infiltrating inflammatory cells, but not epithe-
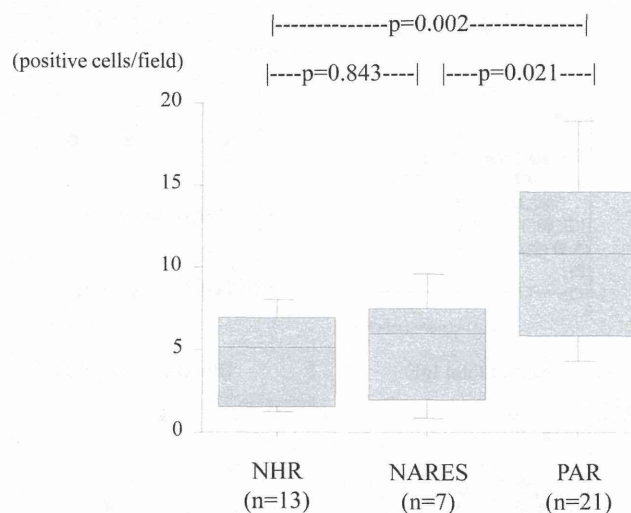


**Figure 2.** *Comparison of numbers of interleukin (IL)-17A+ cells in the nasal mucosa among patients with nonallergic hypertrophic rhinitis (HR), nonallergic rhinitis with eosinophilia syndrome (NARES), and perennial allergic rhinitis (PAR). The rectangle includes the range from the 25th to the 75th percentiles, the horizontal line indicates the median, and the vertical line indicates the range from the 10th to 90th percentiles. The p values were determined by the Mann-Whitney U test.*

lial cells, vascular endothelial cells, glands, or fibroblasts. The expression levels of IL-17A were determined in every group, with a greater expression observed in the PAR group versus the HR and NARES groups (Fig. 1). The number of IL-17A+ cells in the nasal mucosa was significantly higher in the PAR group compared with HR ($p = 0.002$) and NARES ($p = 0.021$) groups. Conversely, the number was similar between the HR and NARES group ($p = 0.843$; Fig. 2).

#### Pathophysiological Significance of IL-17A Expression in Nasal Mucosa

A significant and positive correlation was seen between the number of IL-17A+ cells in the nasal mucosa and total nasal symptom score, which was determined from the sum of sneezing, rhinorrhea, and congestion scores ($\rho = 0.403$; $p = 0.011$; Fig. 3 A). In detail, the sneezing score *per se* was significantly and positively correlated with the number of IL-17A+ cells ($\rho = 0.471$; $p = 0.003$; Fig. 3 B), whereas the rhinorrhea ($\rho = 0.291$; $p = 0.066$; Fig. 3 C) and congestion ($\rho = 0.206$; $p = 0.192$; Fig. 3 D) were not.

The number of IL-17A+ cells did not correlate with total serum IgE levels ($\rho = 0.284$; $p = 0.098$; Fig. 3 E), blood eosinophil counts ($\rho = 0.302$, p = .056, Fig. 3 F), or forced expiratory volume in 1 second/forced vital capacity ratio ($\rho = 0.092$; $p = 0.569$; Fig. 3 G). However, the degree of eosinophil infiltration into the nasal mucosa was significantly and positively
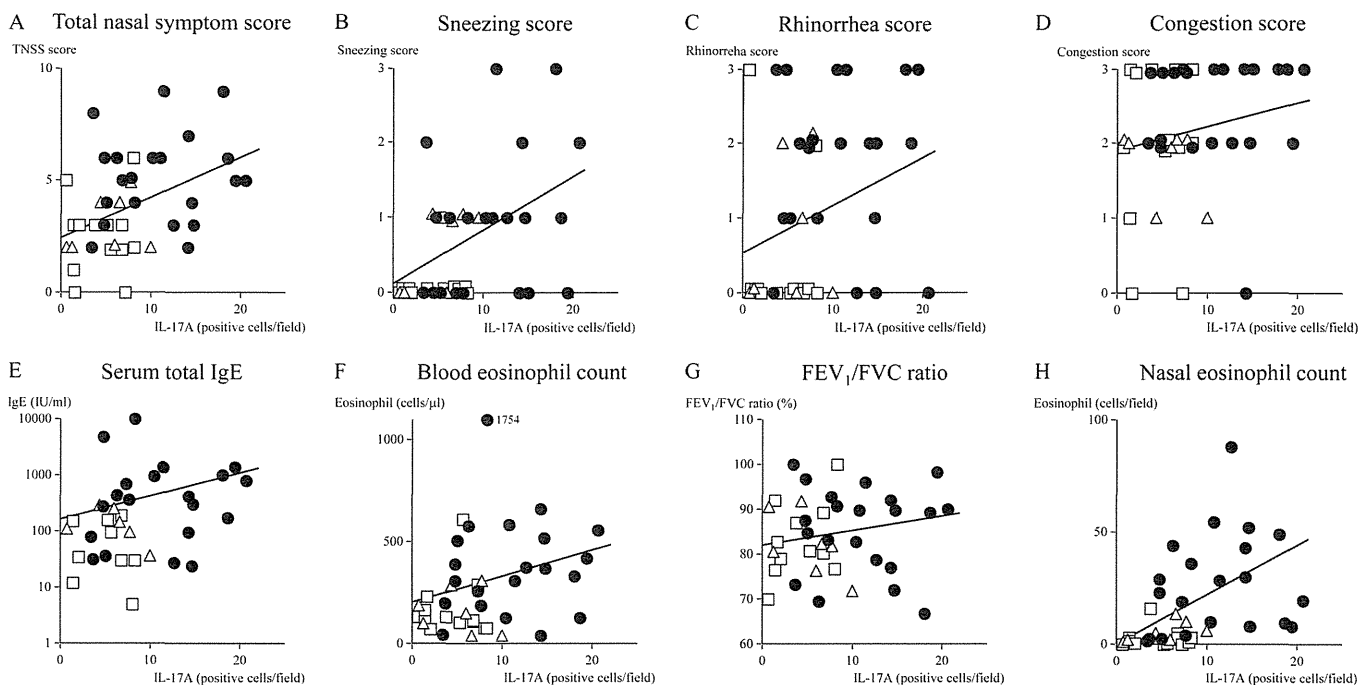
Figure 3. *Relationship between numbers of interleukin (IL)-17A$^+$ cells in nasal mucosa and pathophysiological characterizations including (A) total nasal symptom score (TNSS), (B) sneezing score, (C) rhinorrhea score, (D) congestion score, (E) serum total IgE levels, (F) blood eosinophil count, (G) forced expiratory volume in 1 second/forced vital capacity (FEV$_1$/FVC) ratio, and (H) number of eosinophils in nasal mucosa. Nasal mucosa were sampled from patients with non-allergic hypertrophic rhinitis (HR; □), nonallergic rhinitis with eosinophilia syndrome (△), and perennial allergic rhinitis (PAR; ●).*

correlated with the number of IL-17A$^+$ cells ($\rho = 0.623$; $p < 0.001$; Fig. 3 H).

## DISCUSSION

In the present study, we characterized the expression of IL-17A in the pathogenesis of AR. Our findings suggest that the expression of IL-17A in the nasal mucosa of the inferior turbinate is associated with not only local eosinophilic inflammation, but also severity of nasal symptoms.

Although we and others have previously shown the expression of IL-17A protein in nasal polyps, this was first studied in the English language to clarify the expression in human inferior turbinate mucosa.[10,25,26] We found three Chinese articles (with English abstract), which reported on IL-17, although they had different results and it was unclear whether they assessed IL-17A or other members of the IL-17 cytokine family.[27–29] Ba et al. showed that the number of IL-17$^+$ cells in tissues of AR patients was higher than that of controls ($p < 0.05$). They also showed that the eosinophilic cell count correlated with the number of IL-17$^+$ cells ($r = 0.446$; $p < 0.05$).[27] Interestingly, in a different report, the same authors described that the expression of IL-17 was only apparent in the nasal mucosa of patients with AR.[28] Liu et al. reported that there were no significant differences between AR and nonallergic rhinitis patients in the protein expression of IL-17 in

inferior turbinate tissues.[29] Local IL-17A expression in the sinonasal mucosa is known to have ethnic and/or regional differences.[30] A recent study by Katotomi-chelakis et al. strengthens the importance of studies in the Asian population because there is a difference in cytokine profile in European populations that changes over time.[31] If IL-17 actually refers to IL-17A in these articles, then our findings corroborate those of Ba et al. and suggest that the local expression of IL-17A is elevated in AR patients, especially in Asia.

The expression of IL-17A in the inferior turbinate mucosa was found positively correlated with the degree of local eosinophilia. We previously reported that the number of IL-17A$^+$ cells in sinonasal tissues was positively correlated with the degree of local eosinophilia in CRS.[10] These results suggest that the local expression of IL-17A is associated with eosinophilic inflammation in both AR and CRS. In this study, we also showed that IL-17A directly induces the production of IL-6 and granulocyte macrophage colony-stimulating factor via dispersed nasal polyp cells, both of which are known to promote eosinophilic inflammation.[32,33] Although it remains unclear as to how IL-17A drives eosinophilic inflammation in AR, nasal eosinophilia in AR may be induced via a cytokine orchestration, in which IL-17A is involved. Bachert et al. showed the importance of IgE levels in tissue for the treatment strategy in CRS.[34] An important finding of this

study is that IL-17A$^+$ cells were significantly and positively correlated with the degree of eosinophil infiltration, but not with total serum IgE levels and blood eosinophil counts. This emphasizes the need of counting IgE levels and eosinophils in tissue rather than blood.

The number of IL-17A$^+$ cells in the nasal mucosa was significantly correlated with the total nasal symptom score, suggesting that IL-17A is closely associated with the disease severity of AR. This result was consistent with previous reports showing that serum IL-17A levels and allergen-induced IL-17A mRNA expression correlate with symptom severity, as assessed *via* a visual analog scale and symptom medication score, respectively.[12,14] When we analyzed the individual symptoms separately, only the sneezing score correlated with the number of IL-17A$^+$ cells. The sneezing reflex, which follows an allergen challenge, is primarily a respiratory reflex induced by the interaction between histamine and the H$_1$-receptor at the sensory nerve terminals.[35] Eosinophil infiltration into the nasal mucosa induces a minimal persistent inflammation and the priming effect, both of which can amplify nasal hyperreactivity.[36] Thus, the expression of IL-17A may induce sneezing *via* an indirect enhancement of eosinophilic inflammation. On the other hand, the congestion score did not correlate with IL-17A expression. From an ethical view, it is hard to sample the inferior turbinate mucosa from patients complaining of slight congestion. One of the reasons why the congestion score did not correlate with the IL-17A expression may be because only subjects with medical treatment–resistant, surgery-required swollen inferior turbinates were included in the present study.

In nasal polyps, we performed double immunofluorescence staining and found that CD68$^+$ cells, CD4$^+$ cells, and EG2$^+$ cells expressed IL-17A.[10] Although we could not find such an investigation in the inferior turbinate, both mononuclear and polynuclear cells expressed IL-17A in the immunohistochemistry, suggesting that infiltrating inflammatory cells such as lymphocytes, plasma cells, macrophages, mast cells, and eosinophils may produce IL-17A in AR.

In conclusion, the present study provides evidence that local IL-17A expression is associated with the pathophysiology of AR, including disease severity and nasal eosinophilia. These observations may provide a basis for future therapeutic approaches targeting IL-17A in the management of severe eosinophilic airway diseases, including AR, CRS with nasal polyps, and bronchial asthma.

## ACKNOWLEDGMENTS

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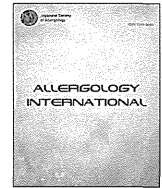
Original article

# Immunological parameters in prophylactic sublingual immunotherapy in asymptomatic subjects sensitized to Japanese cedar pollen

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ARTICLE INFO

A B S T R A C T

Background: This study aims to examine the immunological parameters, focusing IL-10 productivity, in prophylactic sublingual immunotherapy (SLIT) in asymptomatic subjects sensitized to Japanese cedar pollen (JCP).
Methods: This study was conducted as part of a randomized, double-blind, placebo-controlled, multiple center trial, and was performed for two consecutive pollen seasons in 2012 and 2013. The present results were based only on our institution. We recruited 29 participants with specific IgE against JCP of at class 2 and higher levels without history of the pollinosis symptoms at the time of JCP scattering. The SLIT group received standardized JCP extract for five months over the pollen season. We observed and judged development of the symptoms in the pollen season. The percentage of IL-10 producing CD4+ T (Tr1) cells, B cells and monocytes were analyzed by flow cytometry. JCP specific IgE and total IgE were also measured.
Results: The ratio of development of cedar pollinosis was significantly lower in the SLIT group compared to the placebo group in 2013. In 2012, the percentage of circulating Tr1 cells and IL-10 producing monocytes significantly increased in the SLIT group. In 2013, the percentage of circulating Tr1 cells and IL-10 producing B cells increased significantly in the SLIT group. The percentage of circulating IL-10 producing monocytes significantly decreased in the placebo group.
Conclusions: Prophylactic SLIT is effective for prevention of the development of pollinosis. Induction of IL-10 producing T cells, B cells and monocytes is an important mechanism of SLIT for prevention of pollinosis in asymptomatic but sensitized subjects.
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## Introduction

Japanese cedar pollinosis is an allergic disease specific to Japan with a high prevalence estimated to be 26.5%, which has increased by 10% over the past ten years.[1] Seasonal allergic rhinitis induced by cedar pollen takes a chronic course in the majority of middle-aged patients.[2] Remission rarely occurs, especially in the younger generation.

Sublingual immunotherapy (SLIT) is safer than conventional percutaneous antigen-specific immunotherapy, and is the only

treatment which can completely cure the disease. It has been shown that SLIT is effective and safe in the treatment of cedar pollinosis by a randomized, placebo-controlled, double-blind study.[3]

About 20% of asymptomatic subjects sensitized to this pollen develop symptoms in the pollen scattering season.[4] Thus, it is important to prevent the development of pollinosis in these asymptomatic, sensitized subjects. To determine whether SLIT can prevent the development of pollinosis in sensitized subjects who have no history of pollinosis, a randomized, placebo-controlled, double blind trail was carried out over two pollinosis seasons in 2012 and 2013 in multiple facilities in Japan.

The mechanism of action of SLIT is not completely understood. However, IL-10 is critical for the induction of specific T cell tolerance and the increase in IL-10 production by monocytes and T cells during inflammatory responses or after SLIT may influence effector

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cells involved in allergic responses. Increased IL-10 production following specific immunotherapy causes anergy in peripheral T cells, and regulates specific IgE and IgG4 production towards normal IgG4-related immunity.[5] Low IL-10 productivity by monocytes and T cells is closely related to sensitivity to multiple allergens and resistance to allergic diseases.[6,7] Augmentation of constitutive IL-10 production from the immune system is a potential therapeutic approach for allergic disorders. Thus we hypothesized that IL-10 may play an important role in prophylactic SLIT for asymptomatic sensitized subjects.

We also examined the ratio of specific IgE against total IgE (sIgE/tIgE), because the evidence concerning the relationship between this ratio and the efficacy of SLIT is conflicting. Di Lorenzo et al. reported that a high sIgE/tIgE ratio was associated with an effective response in sublingual and subcutaneous immunotherapy in monosensitized patients for the following allergens: grass, Parietaria judaica, Olea europea and house dust mite.[8] On the other hand, Fujimura et al. reported that the sIgE/tIgE ratio before treatment correlated with the symptom-medication score in the SLIT group and that patients with low sIgE/tIgE ratios were more responsive to SLIT in treatment for Japanese pollinosis.[9]

We examined the immunological parameters, including IL-10 productivity, in prophylactic SLIT in asymptomatic subjects sensitized to Japanese cedar pollen.

## Methods

### Study population

This study was conducted as part of a randomized, double-blind, placebo-controlled, multiple center trial in asymptomatic subjects sensitized to Japanese cedar pollen (JCP), and the present results were based only on our institution. The study was performed for two pollen seasons from December 2011 to April 2013. We recruited 29 participants with IgE specific to JCP of at class 2 and higher without history of symptomatic pollinosis during JCP scattering. Japanese cedar pollen-specific IgE titers and total IgE in the serum were measured by CAP-FEIA (fluorescent enzyme immunoassay) (Phadia, Tokyo, Japan) before the study. Participants who were pregnant, breastfeeding or suffering from chronic rhinosinusitis were excluded.

### Ethics statement

This study adhered to the tenets of the Declaration of Helsinki, and was approved by Mie University, Graduate School of Medicine Ethical Committee (No. 2283). A written informed consent was obtained from each subject before study.

### Clinical protocols

The enrolled candidates were randomized into two groups by age and the levels of Cry j 1-specific IgE. The SLIT group received standardized JCP extract (Torii Pharmaceutical Co. Ltd., Tokyo, Japan),[10] and the placebo group received an inactive placebo. The protocol consisted of treatments with graded courses of the extract in 50% glycerol, followed by maintenance therapy.[11] Briefly, the extracts were graded in two concentrations: 200 and 2000 JAU/ml. From early December, the subjects received increasing doses beginning with 0.2 ml of the 200 JAU/ml vial and increasing by 0.2 ml every second day until reaching the maintenance dose of 1.0 ml of the 2000 JAU/ml for two weeks. From the third week, they received the maintenance dose of 1.0 ml of the 2000 JAU/ml daily until the end of April in the following year. The vaccine was taken sublingually, kept for 2 min without a retention reagent and then swallowed. The subjects in the placebo group received inactive 50% glycerol in saline.

### Clinical symptoms and safety measurements

The subjects completed a pollinosis diary to record their nasal and eye symptoms and their use of symptom-reducing drugs. Development of the symptoms was determined on the basis of the pollinosis diary and a nasal provocation test performed at the end of April. The total amounts of pollen scattered from the Japanese cedar and Japanese cypress (Chamaecyparis obtusa) in Tsu city, Mie Prefecture, were 7031 and 16,578 grains/cm$^2$ during 2012 and 2013 pollen seasons, respectively.

### Total and antigen-specific immunoglobulin titer

The levels of Cry j 1-specific IgE and total IgE in serum were measured by CAP-FEIA (fluorescent enzyme immunoassay) (Phadia, Tokyo, Japan).

### Blood samples and PBMC culture

Peripheral blood was obtained from each subject before and after treatment (December and April) each year. Peripheral blood mononuclear cells (PBMC) were isolated from 10 ml of heparinized venous blood by density gradient centrifugation using Ficoll 1077 (Sigma, St. Louis, MO, USA). PBMC were cultured in RPMI 1640 medium (Nikken Bio Medical Laboratory, Kyoto, Japan) containing L-glutamine supplemented with 100 U/mL penicillin, 100 U/mL streptomycin (Invitrogen, Carlsbad, CA, USA) and 10% Human AB serum (Gemini Bio-Products, West Sacramento, CA, USA). Cells were plated onto 24-well tissue culture plates at a density of $2 \times 10^6$ cells/mL/well and were incubated with 10 JAU of Cry j1 (Torii, Tokyo, Japan) for 8 h at 37 °C in an atmosphere of 5% CO$_2$. Endotoxin level was confirmed to be less than 0.1 ng/μg (1 EU/μg) of the protein in Cry j1.

### IL-10 staining in T cells, B cells and monocytes

After 8 h cultivation with antigens, PBMC were collected and incubated with PE-conjugated IL-10 secretion assay kit according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA). Cells were also co-stained with anti-CD4-FITC and CD19-PECy5 antibodies, or anti-CD14-FITC antibody (eBioscience, Sam Diego, CA, USA). The percentage of IL-10 producing CD4$^+$ T cells, B cells and monocytes were determined using an Accuri C6 flow cytometer (Becton Dickinson, Mansfield, MA).

### Statistical analysis

Two-group comparisons were performed using a Wilcoxon test or Mann–Whitney U-test to determine the significance of differences, or using an unpaired t-test as indicated. A p value of less than 0.05 was considered statistically significant.

## Results

### Clinical effects

Two subjects withdrew during the course of the study. The demographic characteristics of the 27 subjects before treatment are shown in Table 1. Clinical data from participants in 2012 and 2013 are shown in Table 2. As shown in Table 1, mean age of placebo group is higher compared to that of SLIT group; however, IL-10 production form T cells or monocytes is unchanged among

younger generations (data not shown). In each year, 17 subjects participated in the study. Seven of the participants in 2012 continued to participate in the second year. These seven subjects did not develop symptoms in the first year.

In 2012, four of the SLIT group and one of the placebo group developed symptoms. There was no significant difference in the ratio of symptom development between the two groups in 2012. In 2013, seven of the placebo group but none of the SLIT group developed symptoms. The ratio of the development of pollinosis in the SLIT group was significantly lower than that of the placebo group in 2013 ($p = 0.0098$, Fisher's exact test). To clarify the possible factors influencing clinical effects, we compared age, sex ratio and sIgE/tIgE ratio between 2012 and 2013. However, there was no significant difference in these factors in the two years (Table 2). We also compared these factors between the SLIT group and the placebo group in each year, but statistical significance was not detected (data not shown).

*Prognostic biomarker for clinical effects*

A comparison of the ratio of sIgE/tIgE in the preseason period is shown in Fig. 1. The ratio of sIgE/tIgE in the preseason was higher in those who developed symptoms in 2013 (B), but not in 2012 (A). In the SLIT group, the sIgE/tIgE ratio in the preseason period was significantly higher in those who developed symptoms (C). In the placebo group, there was no significant difference in the ratio between the subjects developed pollinosis and those did not (D).

*IL-10 production in T cells, B cells and monocytes during SLIT*

In 2012, it was impossible for us to collect blood from one subject in each group due to their personal reasons. In 2013, one subject as the first year trial in the SLIT group and another subject as the second year trial in the placebo group were unable to come to the hospital for the blood collection in the postseason due to their personal reasons. Thus, we were unable to obtain blood samples from four subjects in total.

The percentage of IL-10-producing T cells, B cells and monocytes in 2012 is shown in Fig. 2. The percentage of circulating Tr1 cells (IL-10$^+$CD4$^+$ T cells/CD4$^+$ T cells) and IL-10 producing monocytes (IL-10$^+$CD14$^+$cells/CD14$^+$cells) significantly increased in the SLIT group ($p = 0.0117$, Tr1 and $p = 0.0117$, monocyte, respectively). However, the number of IL-10 producing B cells (IL-10$^+$CD19$^+$cells/CD19$^+$cells) remained unchanged. The results for the 2013 season are shown in Fig. 3. The percentages of circulating Tr1 and IL-10 producing B cells significantly increased in the SLIT group ($p = 0.0277$, Tr1 and $p = 0.0277$, B cell, respectively). On the other hand, the percentage of circulating IL-10 producing monocytes significantly decreased in the placebo group ($p = 0.0077$).

**Table 1**
Clinical data of 27 participants at the start of the study.

| Group | SLIT | Placebo | P-value |
|---|---|---|---|
| Number | 13 | 14 | |
| Male/Female | 5/8 | 7/7 | N.S.[†] |
| Age (mean, range) | 27.8 (18–47) | 36.1 (19–52) | $p = 0.03$[‡] |
| Total IgE (mean, range) | 214 (23–823) | 222 (5–1310) | N.S.[‡] |
| Specific IgE[§] (mean, range) | 13.9 (0.74–84.7) | 9.64 (0.88–49.3) | N.S.[‡] |
| Class (mean, range) | 2.8 (2–5) | 2.6 (2–4) | N.S.[‡] |
| Ratio of sIgE/tIgE (mean, range) | 0.11 (0.0049–0.44) | 0.15 (0.00061–0.63) | N.S.[‡] |

[†] Yates 2 × 2 Chi-squared test.
[‡] Student t-test.
[§] Specific IgE to Japanese cedar pollen; ImmunoCAP raw value [UA/ml], mean.

**Table 2**
Clinical data of participants in 2012 and 2013.

| Group | 2012 | 2013 | P-value |
|---|---|---|---|
| Number | 17 | 17 | |
| SLIT/placebo | 9/8 | 7/10 | |
| Male/Female | 7/10 | 9/8 | N.S.[†] |
| Age (mean, range) | 34.2 (21–52) | 34.6 (18–52) | N.S.[‡] |
| Total IgE (mean, range) | 121 (22.5–548) | 298 (5.0–1310) | N.S.[‡] |
| Specific IgE[§] (mean, range) | 11.7 (1.2–84.7) | 10.2 (0.7–49.3) | N.S.[‡] |
| Class (mean, range) | 2.8 (2–5) | 2.8 (2–4) | N.S.[‡] |
| Ratio of sIgE/tIgE (mean, range) | 0.15 (0.0061–0.49) | 0.11 (0.0048–0.63) | N.S.[‡] |

[†] Yates 2 × 2 Chi-squared test.
[‡] Student t-test.
[§] Specific IgE to Japanese cedar pollen; ImmunoCAP raw value [UA/ml], mean.

The enrolled patients in 2013 were divided into two categories: the subjects with first year trial and the second year trial. Therefor the data of IL-10-producing T cells, B cells and monocytes in 2013 was calculated separately. As shown in Fig. 4A, nine subjects had participated as the first year trial. Three subjects in the SLIT group and six subjects in the placebo group are shown. IL-10 producing monocytes were statistically decreased in placebo group at the post season. The other hand, six subjects had participated as the second year trial. Three subjects in the SLIT group and three subjects in the placebo group are shown. Although the significant difference was undetected, the actual percentage of Tr1 and IL-10 producing B cells seems to be higher in the second-year trial compared to the first-year trial (Fig. 4B).

## Discussion

To our knowledge, this is the first report examining the percentage of circulating IL-10 producing T cells, B cells and monocytes in prophylactic SLIT treatment in those who are sensitized to pollen but do not develop pollinosis. A significant increase in the percentage of Tr1 in the SLIT group was observed in both years of the study. The up-regulation of Tr1 cells may play a critical role in specific immunotherapy and be a useful marker of successful response in allergic rhinitis patients. It has been reported that the levels of allergen-specific Tr1 cells, IgG4 and allergen-induced IL-10 synthesis from PBMC cultures were significantly increased after cluster specific immunotherapy in Der p-sensitized children with allergic rhinitis compared with baseline levels, with a significant correlation between increased levels of Tr1 cells and improvement in nasal symptoms.[12]

A significant increase in the circulating IL-10 producing B cells in the SLIT group was observed only in the 2013 season. IL-10-producing regulatory B cells suppress immune responses, and the lack of these cells leads to exacerbated symptoms. According to van de Veen et al., IL-10 producing B cells suppressed antigen-specific CD4$^+$ T-cell proliferation.[13] B cells specific for the major bee venom allergen phospholipase A2 (PLA) isolated from non-allergic beekeepers show increased expression of IL-10 and IgG4. IgG4 is a blocking antibody isotype with anti-inflammatory potential that is induced in human high-dose antigen tolerance.[13] Furthermore, the frequency of IL-10$^+$ PLA specific B cells increased in allergic patients receiving allergen-specific immunotherapy.[13] IL-10-producing B cells, also known as regulatory B cells (B regs), also play a key role in controlling autoimmunity. Mice lacking endogenous IL-10-producing regulatory B cells developed exacerbated disease and presented with a decrease in regulatory T cells.[14]

Monocyte is a major source of IL-10 in PBMC, and is a key cells for IL-10 mediated immunomodulation. The changes in percentage of IL-10 producing monocytes were different between the two
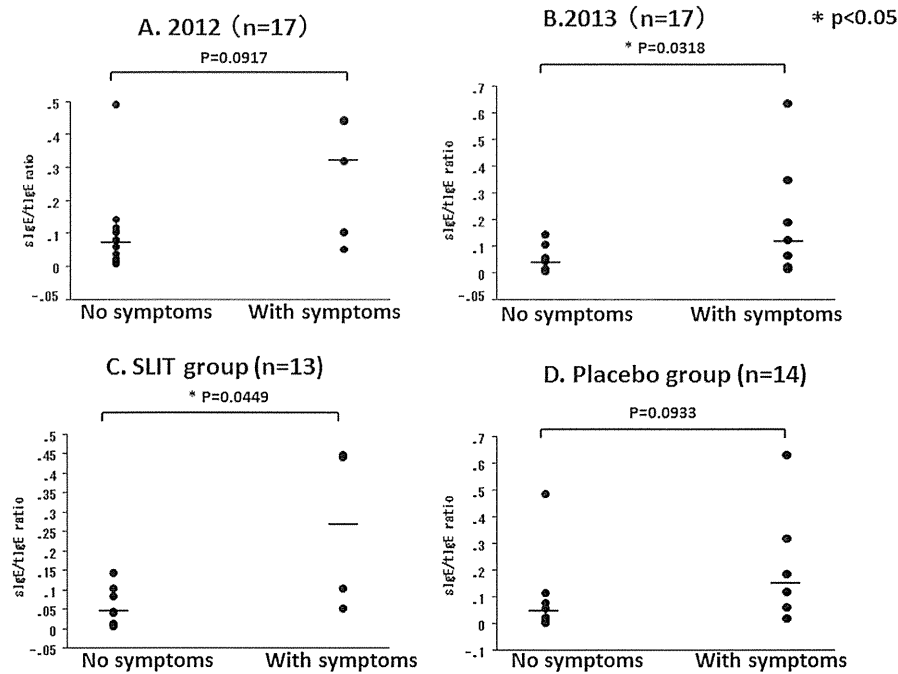
## A. 2012 (n=17)

P=0.0917

## B.2013 (n=17)     * p<0.05

* P=0.0318



## C. SLIT group (n=13)

* P=0.0449

## D. Placebo group (n=14)

P=0.0933



**Fig. 1.** Comparison of the ratio of sIgE/tIgE in the preseason period. The ratio of sIgE/tIgE in the preseason was higher in those who developed symptoms in 2013 (B), but not in 2012 (A). In the SLIT group, the sIgE/tIgE ratio in the preseason period was significantly higher in those who developed symptoms (C). In the placebo group, there was no significant difference in the ratio between those who developed pollinosis and those who did not (D). When the subjects received SLIT for two years, only the data in the first year was adopted. The graph shows the mean ± standard deviation. The comparison was made using Mann–Whitney U-test.

years. In 2012, the percentage of IL-10 producing monocytes significantly increased in the SLIT group. On the other hand, the percentage of IL-10 producing monocytes significantly decreased in the placebo group in 2013. Therefore a decrease in the percentage of IL-10 producing monocytes was prevented with the SLIT protocol. Pollinosis developed in 70% of the placebo group but none of the SLIT group in 2013. The percentage of IL-10 producing monocytes was significantly higher in healthy subjects than in those
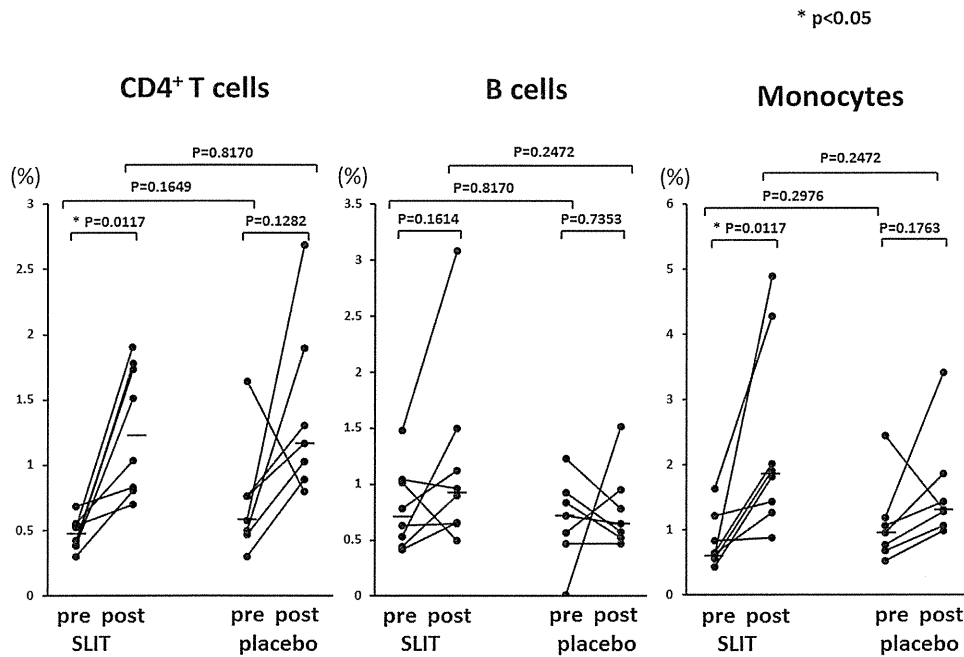
* p<0.05

## CD4$^+$ T cells

## B cells

## Monocytes



**Fig. 2.** The percentage of IL-10-producing T cells, B cells and monocytes in 2012. The percentage of circulating Tr1 (IL-10$^+$CD4$^+$ T cells/CD4$^+$ T cells) cells significantly increased in the SLIT group. The percentage of circulating IL-10 producing monocytes (IL-10$^+$CD14$^+$cells/CD14$^+$cells) significantly increased in the SLIT group. However, the number of IL-10 producing B cells (IL-10$^+$CD19$^+$cells/CD19$^+$cells) remained unchanged. ($n = 8$ in SLIT, $n = 7$ in placebo) The comparison between the pre-season values and the post-season values was performed using a Wilcoxon test. The comparison between the SLIT group and the placebo group was done using the Mann–Whitney U-test.
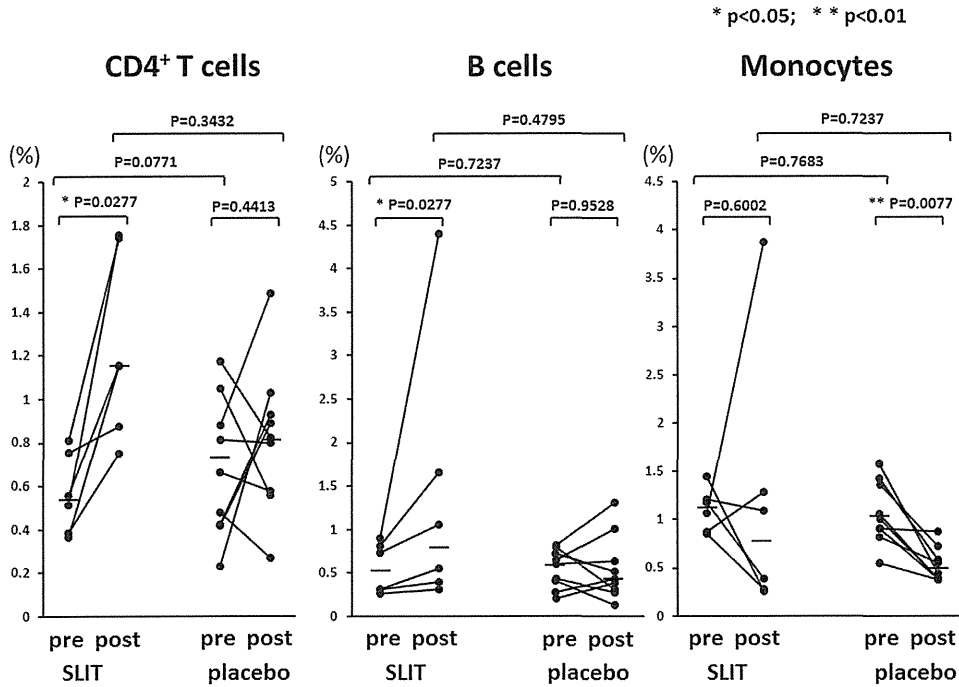
* p<0.05;  * * p<0.01



**Fig. 3.** The percentage of IL-10-producing T cells, B cells and monocytes in 2013. The percentage of circulating Tr1 (IL-10$^+$CD4$^+$ T cells/CD4$^+$ T cells) cells and IL-10 producing B cells (IL-10$^+$CD19$^+$cells/CD19$^+$cells) significantly increased in the SLIT group. The percentage of circulating IL-10 producing monocytes (IL-10$^+$CD4$^+$cells/CD14$^+$cells) significantly decreased in the placebo group. ($n$ = 6 in SLIT, $n$ = 9 in placebo) The comparison between the pre-season values and the post-season values was performed using a Wilcoxon test. The comparison between the SLIT group and the placebo group was performed using the Mann–Whitney U-test.
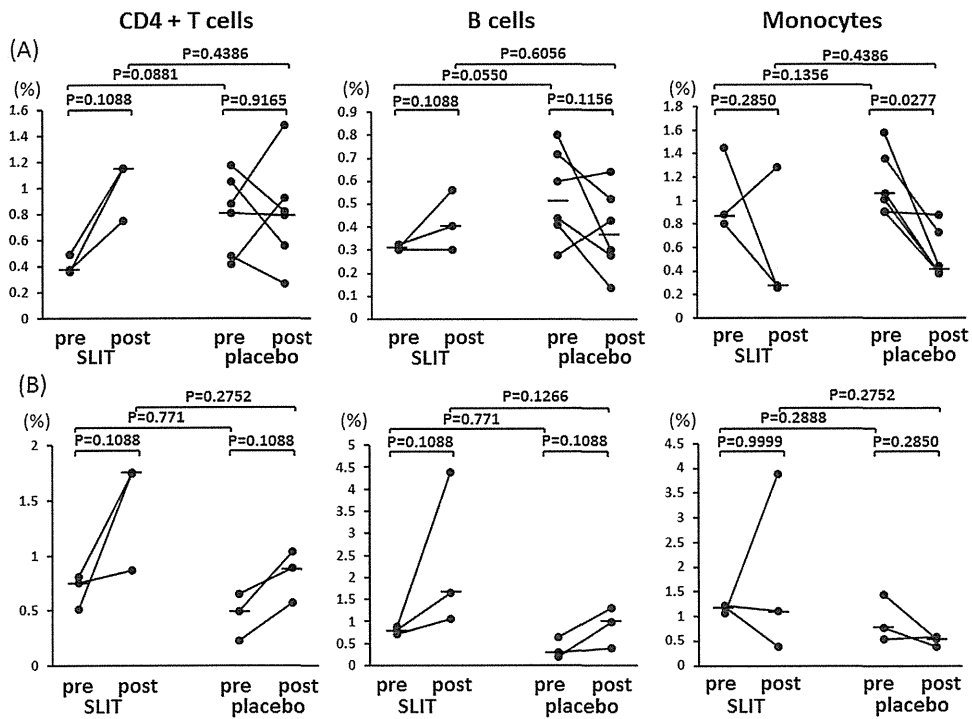


**Fig. 4.** The percentage of IL-10-producing T cells, B cells and monocytes in 2013. (A) This figure shows 10 subjects who have participated in the first year. Three subjects in the SLIT group and six subjects in the placebo group are shown. IL-10 producing monocytes were statistically decreased in placebo group at the post season. (B) This figure shows 7 subjects who have participated in two consecutive years. Three subjects in the SLIT group and three subjects in the placebo group are shown. The significant difference was undetected. The comparison between pre-season values and the post-season values was performed using a Wilcoxon test. The comparison between the SLIT group and placebo group was performed using the Mann–Whitney's U-test.

suffering Japanese cedar pollinosis.[6] Thus from the result in 2013, it is assumed that decreased IL-10 production by monocytes is closely related to becoming symptomatic. On the other hand, SLIT prevented subjects from becoming symptomatic by up-regulating the IL-10 productivity. Monocytes recognize antigens via Toll like receptors, digest antigen with intrinsic enzyme, and some populations such as CD14+CD16+ monocytes present antigens combined with expression of MHC class II like antigen presenting cells.[15]

The reason for the difference in the clinical efficacy of SLIT and the percentage of IL-10 producing cells between the two years is unclear. In 2012, nearly half of subjects received SLIT treatment developed the symptoms, although IL-10 producing T cells significantly increased in the SLIT group. Among patients with symptomatic pollinosis treated by SLIT, some patients do not show the efficacy, although Tr1 ratio is increased. In the same way, in 2012 we speculate increased level of Tr1 induced by SLIT was not enough to suppress nasal allergy symptoms. The difference in the exposed pollen amounts might have some effects to the results. Exposure to larger amounts of pollen in 2013 might have helped switching and expansion of IL-10 producing cells. It is known that high dose bee venom exposure in beekeepers by natural bee stings represents a model to understand mechanisms of T cell tolerance to allergens in healthy individuals.[16] Rapid switch and expansion of IL-10-producing Tr1 cells and the use of multiple suppressive factors represent essential mechanisms in immune tolerance to a high dose of allergens in non-allergic individuals.[16]

The subjects who developed pollinosis in 2012 did not participate in the study the next year. Thus, the study in 2013 comprised new subjects ($n = 10$) and those who did not develop pollinosis in 2012 ($n = 7$). This might have influenced the clinical efficacy of SLIT and the percentage of the IL-10 producing cells.

Those who developed pollinosis in the SLIT group had a significantly higher sIgE/tIgE ratio which supports the results reported by Fujimuara et al.[9] We speculate that effector cells with a low specific IgE level are less likely to be activated by antigen crosslinking or are more susceptible to downregulation by IL-10 producing cells than those with a high specific IgE level. The symptoms of patients with a low sIgE/tIgE ratio may be more easily attenuated by suboptimal potentiation of IL-10 producing cells by SLIT.[9] A high sIgE/tIgE ratio before the season may be predictive of development of pollinosis. Uekusa et al.[4] examined 33 adults who were sensitized to JCP but who had not developed symptoms and found that the sIgE/tIgE was significantly higher before the season in the subjects who developed pollinosis.

A limitation of the present study is the small number of samples. Further study is necessary to perform the analysis using a larger number of subjects.

Prophylactic SLIT is effective in preventing development of pollinosis. IL-10 producing T cells, B cells and monocytes play important roles in the mechanism of SLIT for the prevention of pollinosis in asymptomatic and sensitized subjects.

## Acknowledgments

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ORIGINAL ARTICLE

# Measurement of Japanese Cedar Pollen-Specific IgE in Nasal Secretions

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## ABSTRACT

**Background:** Japanese cedar pollen (JCP) is the most common allergen for seasonal allergic rhinitis in Japan. Little is known about local production of immunoglobulin (Ig)E in people with or without Japanese cedar pollinosis. The aims of this study were to measure levels of JCP-specific IgE in nasal secretions and determine correlations with levels in serum.

**Methods:** Forty-six subjects were enrolled in this study, comprising 24 symptomatic subjects, 9 asymptomatic subjects sensitized to JCP, and 13 subjects not sensitized to JCP. Nasal secretions were obtained during a period of Japanese cedar dispersal, and levels of JCP-specific IgE were measured with CAP-fluorescent enzyme immunoassay. Serum JCP-specific IgE and total IgE were also measured using the same method.

**Results:** Among the 46 subjects enrolled, JCP-specific IgE in nasal secretions was measureable in 43 subjects. Irrespective of symptom development, sensitized subjects showed higher levels of JCP-specific IgE in nasal secretions than non-sensitized subjects. A significant moderate correlation was observed between JCP-specific IgE levels in nasal secretions and serum in all 43 subjects. With stratification by subject group, only symptomatic subjects showed a substantial correlation between JCP-specific IgE levels in nasal secretions and serum.

**Conclusions:** Our results imply a certain association between JCP-specific IgE in nasal secretions and sensitization of Japanese cedar pollinosis. Therefore, levels of allergen-specific IgE in nasal secretions can be used as an alternative diagnostic marker for allergic rhinitis patients.

## KEY WORDS

allergen-specific IgE, allergic rhinitis, diagnostic marker, Japanese cedar pollinosis, nasal secretion

## INTRODUCTION

Allergic rhinitis (AR) is an immunoglobulin (Ig)E-mediated inflammatory disease of the nasal mucosa resulting from exposure to an inhalant allergen. IgE plays an important role in the development of AR. One of the ways to determine the presence of sensitization to allergens is to measure allergen-specific IgE levels in serum, and this approach is used widely in daily practice. Many studies have detected IgE not only in serum, but also in nasal secretions in patients with AR,[1,2] showing local production of IgE in the nasal mucosa. Recent research has shown local production of IgE in patients who are negative for serum IgE, leading to the concept of local AR.[3-8] Local IgE production is thought to play a more important role

than IgE in the serum in allergic inflammation.

The origin of allergen-specific IgE in nasal secretions has been addressed by many studies. In patients with AR, allergen-specific IgE is detected in nasal mucosa[9] in addition to nasal secretions.[2] Moreover, somatic hypermutation and class switching to IgE have been observed in the nasal mucosa in patients with AR,[10] suggesting that locally detected IgE is produced in nasal mucosa rather than a mere transudate from serum. Until recently, the major sources and sites of allergen-specific IgE production have not been fully identified. However, a recent study showed that the majority of allergen-specific IgE in peripheral blood is not derived from IgE-secreting cells in the blood, suggesting local IgE production in tissues as a major source of allergen-specific IgE.[11] Given such

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findings, allergen-specific IgE in both nasal secretions and serum may be derived from common sites of production.

Allergens vary from one region to another. In Japan, Japanese cedar pollen (JCP) from *Cryptomeria japonica* represents the most common allergen for seasonal AR, affecting more than 30% of the population as Japanese cellar pollinosis, and the morbidity rate has been rising.[12] Despite many clinical and basic investigations into Japanese cellar pollinosis, little is known about local production of IgE in individuals with or without Japanese cedar pollinosis. No standardized method for quantitative measurement of IgE in nasal secretions is clinically available. Furthermore, the correlation between concentrations of IgE in serum and in nasal secretions has not been elucidated. The aims of this study were both to measure JCP-specific IgE in nasal secretions using a clinically available method and to evaluate correlations with its serum counterpart in subjects with or without Japanese cedar pollinosis.

## METHODS

### STUDY SUBJECTS
A total of 46 subjects (age range, 1-78 years) with or without Japanese cedar pollinosis were recruited in the present study. These included healthy subjects as well as patients who were under treatment for various diseases in the outpatient clinic of our department. Patients with chronic rhinosinusitis were excluded from this study.

### STUDY DESIGN
The study was performed from January to March 2012, during a period of JCP dispersal in Japan. Symptoms associated with allergic rhinitis and levels of JCP-specific IgE in both serum and nasal secretions were evaluated. Medication was not restricted for subjects in this study. During the study period, subjects took medications as usual for their underlying medical conditions, including Japanese cedar pollinosis.

Subjects were classified into the following 3 groups according to sensitization to JCP and presence of symptoms of Japanese cedar pollinosis: Group 1, which included symptomatic subjects who were sensitized to JCP; Group 2, which included asymptomatic subjects who were sensitized to JCP; and Group 3, which included asymptomatic subjects who were not sensitized to JCP. The presence of Japanese cedar pollinosis was defined as development of typical symptoms affecting the nose and eyes during JCP dispersal and eosinophilia in nasal secretions. Sensitization was defined as serum JCP-specific IgE levels ≥0.70 kUA/mL (IgE ImmunoCAP class ≥2).

This study was approved by the ethics committee of Mie University. Written informed consent was obtained from all subjects and parents or guardians of subjects under 20 years old.

## PREPARATION OF NASAL SECRETIONS FOR SPECIFIC IgE MEASUREMENT
Nasal secretions were obtained using an ATOMS tap® (Lumenis, Tokyo, Japan). The ATOMS tap® is a device originally designed for collecting middle ear effusion for the diagnosis of otitis media. The instrument is equipped with a suction tip about 2 mm in diameter and 60 mm in length, and a bottle into which the aspirated sample is collected. The tip was inserted into the nasal cavity of subjects to aspirate nasal secretions. The obtained nasal secretions were frozen at -20℃ until analysis. After thawing, the nasal secretions were diluted with 0.05 M phosphate saline buffer. Samples were centrifuged for 5 min, and supernatants were collected for measurement of IgE.

## MEASUREMENT OF TOTAL AND SPECIFIC IgE TITERS OF SERUM AND NASAL SECRETIONS
Levels of JCP-specific IgE in nasal secretions and serum were measured with CAP-fluorescent enzyme immunoassay (FEIA) (Phadia, part of Thermo Fisher Scientific, Tokyo, Japan) in the same manner used in daily practice. Total IgE in serum was also measured this way.

## SPIKE AND RECOVERY TESTS
Nasal secretion contains many substances including IgA and mucin, which may interfere with the assay. To validate and assess the accuracy of the assay, spike and recovery tests were performed as follows. Among the set of the samples of nasal secretions preserved, 4 samples whose concentrations of JCP-specific IgE had been determined below 0.1 U/mL were selected and thawed. The concentrations of IgE of the samples were measured and found to be identical to those measured before preservation. A human serum containing high concentration of IgE was used as a spiking solution. The spiking solution was added to each sample at 1 : 10 dilution. The spiking solution was also added to the standard diluent (0.05 M phosphate saline) at the same dilution. The standard dilute has been proven not to interfere with the assay. The concentrations of IgE in each spiked sample and the spiked standard diluent were determined. The recovery rate was calculated as the ratio of the value of each spiked sample to that of the spiked standard dilute.

## LINEARITY OF DILUTION TESTS
Among the set of the samples of nasal secretions, a sample with high concentration of JCP specific IgE was selected. The sample was serially diluted with the standard dilute to make dilution 1 : 2, 1 : 4, 1 : 8, and 1 : 16. The concentrations of IgE in each diluted sample were measured. The recovery rate was calculated as the ratio of measured values at each dilution

**Table 1** Epidemiological and laboratory data of study subjects

| Group | Group 1 | Group 2 | Group 3 |
|---|---|---|---|
| Subjects enrolled (*n*) | 24 | 9 | 13 |
| Subjects analyzed (*n*) | 23 | 8 | 12 |
| Age | 49 ± 16 | 34 ± 11 | 48 ± 22 |
| Sex, *n* (%) | | | |
| Male | 13 (57%) | 3 (38%) | 5 (42%) |
| Female | 10 (43%) | 5 (62%) | 7 (58%) |
| IgE | | | |
| Positive detection rate | 78%[†] | 63% | 17% |
| JCP in nasal secretions (kUA/mL) | 4.9 ± 9.9[†] | 0.50 ± 0.51 | 0.20 ± 0.49 |
| JCP in serum (kUA/mL) | 25 ± 28[†] | 9.1 ± 9.0[‡] | $0.3 ± 1.3 × 10^{-7}$ |
| Total IgE in serum (kU/mL) | 247 ± 392 | 119 ± 93 | 79.0 ± 90.4 |

Data are expressed as mean ± standard deviation.

JCP, Japanese cedar pollen; JCP in nasal secretions, IgE specific to JCP in nasal secretion; JCP in serum, IgE specific to JCP in serum.
[†] Significant difference between Groups 1 and 3. [‡] Significant difference between Groups 2 and 3.

to the expected values deduced based on the given respective dilution. A linear regression analysis was carried out to determine the extent of the relationship between the measured values and the expected values.

## STATISTICAL ANALYSIS

Spearman's rank correlation coefficient was used to evaluate correlation between two sets of variables. Data among the 3 groups were compared using the Kruskal-Wallis test. If a significant difference was found, post-hoc comparisons were carried out by the Mann-Whitney U test with Bonferroni's correction. The chi-square test was used to examine differences with categorical variables. Values of $P < 0.05$ were considered significant. All statistical analyses were carried out using JMP version 5.1.1 software (SAS Institute, Cary, NC, USA).

## *RESULTS*

### SUBJECTS

The 46 subjects comprised 22 males and 24 females, with 24 subjects in Group 1, 9 in Group 2, and 13 in Group 3.

### SUBJECT CHARACTERISTICS AND LABORATORY DATA

Table 1 shows the details of subject characteristics and all data concerning IgE. Of the 46 samples of nasal secretions obtained from 46 subjects, levels of JCP-specific IgE were successfully measured in 43. Measurement was technically impossible in remaining 3 samples due to excessively high viscosity, and these 3 subjects were subsequently eliminated from the analysis. As a result, Groups 1, 2, and 3 included 23, 8, and 12 subjects, respectively. No complications were seen in any subjects during the collection of na-

sal secretions. Of the 43 samples successfully measured, JCP-specific IgE was detected in 58%. Detection rates in each group were 78%, 63%, and 17% for Groups 1, 2, and 3, respectively.

Levels of JCP-specific IgE in nasal secretions were then compared among the 3 groups (Fig. 1). Group 1 showed significantly higher levels of JCP-specific IgE in nasal secretions than Group 3; however, no significant difference was observed between Groups 1 and 2. Additionally, no significant difference was observed between Groups 2 and 3. Levels of JCP-specific IgE in serum were also compared among the 3 groups. Groups 1 and 2 each showed significantly higher serum levels of JCP-specific IgE than Group 3, but no significant difference was observed between Groups 1 and 2. Additionally, no significant difference was observed in total IgE among the 3 groups (Table 1).

## CORRELATIONS BETWEEN JCP-SPECIFIC IgE IN NASAL SECRETIONS AND JCP-SPECIFIC AND TOTAL IgE IN SERUM

A significant moderate association was observed between JCP-specific IgE in nasal secretions and JCP-specific IgE in serum overall for the 43 subjects ($r = 0.79$, $P < 0.001$) (Fig. 2A). When all data were stratified by subject group and analyzed separately, a significant positive correlation was observed only for Group 1 ($r = 0.78$, $P < 0.001$) (Fig. 2B). No significant correlations were seen for Groups 2 and 3 (Fig. 2C, D). A similar significant moderate association was observed between JCP-specific IgE in nasal secretions and total IgE in serum in all 43 subjects ($r = 0.65$, $P < 0.001$).

## SPIKE AND RECOVERY TESTS

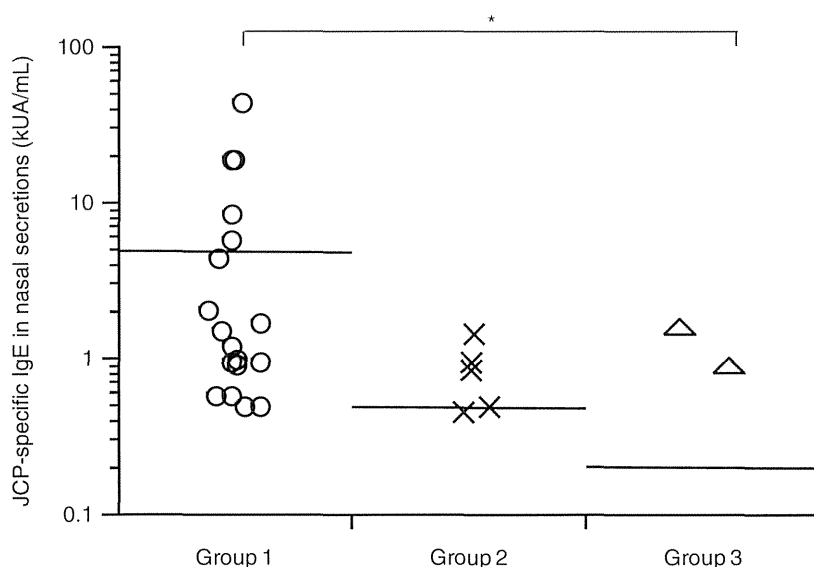The recovery rates of each sample were 102%, 105%, 108%, and 109%.

**Fig. 1** Comparison of JCP-specific IgE levels in nasal secretions among the 3 groups. Individual data are shown. Since the majority of values from the 3 groups were clumped at the bottom of the graph when data were shown on a linear scale, data are shown on a logarithmic scale to make the graph more understandable. Each dot is shown if its value is greater than zero, because zero cannot be shown on the log scale. Horizontal bars represent means for each group. *$P$ < 0.005.

## LINEARITY OF DILUTION TESTS

The recovery rates of each sample at dilution of 1 : 2, 1 : 4, 1 : 8, and 1 : 16 were 106%, 106%, 110%, and 119% respectively. Adjusted coefficient of determination was 0.999 and the equation obtained was: $y = 0.159 + 1.04x$, where $y$ was the measured values and $x$ was expected values. Coefficient correlation was 0.999 and $P$-value was below 0.001.

## DISCUSSION

This study was intended to measure allergen-specific IgE in nasal secretions in patients with allergic rhinitis caused by JCP. It had two major findings. First, JCP-specific IgE was detected in most symptomatic subjects and in more than half of asymptomatic but sensitized subjects, and levels of JCP-specific IgE both in nasal secretions and in serum correlated significantly in symptomatic subjects. Second, nasal secretions were able to be obtained easily and safely using a simple suctioning and collection device.

JCP-specific IgE was detected in most Group 1 (symptomatic) subjects. Previous studies have detected allergen-specific IgE in nasal secretions in patients with AR induced by several aeroallergens.[1,2,13,14] The high detection rate of JCP-specific IgE in nasal secretions from symptomatic patients in our study was consistent with those results. In addition, our study showed that 63% of Group 2, which represented asymptomatic but sensitized subjects, had JCP-specific IgE in nasal secretions. To the best

of our knowledge, analysis of allergen-specific IgE in nasal secretions from asymptomatic sensitized subjects has not been reported previously. Furthermore, although levels were quite low, a small portion of Group 3, representing asymptomatic and non-sensitized subjects, also showed positive IgE in nasal secretions. A possible explanation for this observation in Group 3 is that JCP-specific IgE may have been present in serum at levels below the threshold of detection. Another possibility is production of JCP-specific IgE only in the nasal mucosa. However, no definite explanation for this observation can be drawn.

A significant moderate correlation between the level of allergen-specific IgE in nasal secretions and that in serum was observed for the overall subject population in our study. With stratification by subject group, only Group 1 showed a significant correlation, representing a novel finding in this field. The overall correlation might be attributable to the high correlation in Group 1, particularly since Group 1 included 53% of all subjects. Generally, quantitative specific IgE levels in serum have been shown to be useful in predicting the probability of AR, and specific IgE serum concentration is associated with symptom severity in children with seasonal AR.[15] Our data imply the possible utility of measuring allergen-specific IgE in nasal secretion as an alternative diagnostic biomarker of allergen-specific IgE in serum. On the other hand, although JCP-specific IgE was detected from nasal se-
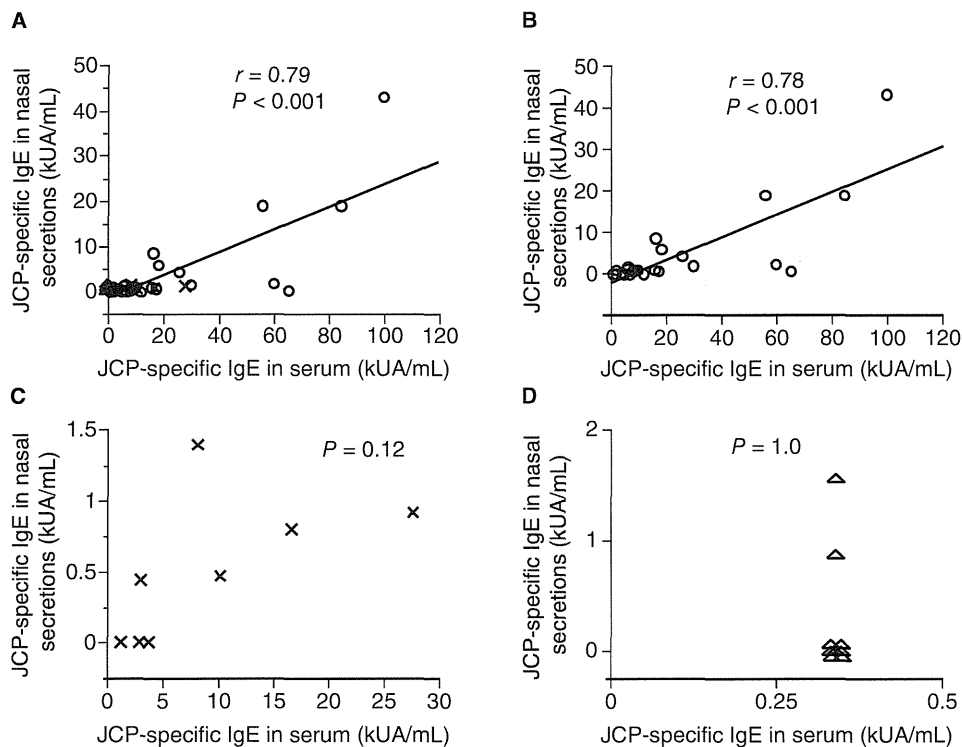
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**Fig. 2** Correlation between JCP-specific IgE in nasal secretions and serum in all subjects and within each group. **A)** All subjects; **B)** Group 1; **C)** Group 2; **D)** Group 3. *P* values are shown. Values for the coefficient of correlation and trend lines are displayed for statistically significant correlations. In Graph D, six dots with values of 0 on the X-axis and 0.34 on the Y-axis overlapped exactly; these dots are jittered slightly for the purposes of depiction.

cretions in 63% of subjects in Group 2, no significant correlation was seen between the level of JCP-specific IgE in nasal secretions and that in serum. It is possible to make a speculative hypothesis as follows on the basis of those observations: JCP-specific IgE is secreted into nasal secretions at a certain concentration if a patient is sensitized to JCP. After developing symptoms of AR, much more JCP-specific IgE is released into nasal secretions, reaching a level corresponding to the level in serum. Since our study was conducted on a practical clinical basis, detailed cellular and molecular mechanisms are beyond the reach of this study.

Second, nasal secretions were obtained easily and safely using a simple suctioning and collection device, the ATOMS tap®. Unlike measurement of allergen-specific IgE in serum, standard methods for collecting nasal secretions and measuring levels of allergen-specific IgE in nasal secretions have yet to be established. Several methods have been employed in the literature to detect IgE in nasal secretions. For instances, some authors have collected nasal secretions by nasal lavage,[7] while others have employed a device using the allergen-coupled cellulose derivative for measurement of IgE.[13,14] We used the ATOMS

tap® in the present study to obtain nasal secretions. The ATOMS tap® is a device originally designed for collecting middle ear effusion for the diagnosis of otitis media and has received formal approval for use as a medical device in Japan. By simply connecting the unit to a conventional suction device, it is instantly ready for use. The tip is thin enough to be inserted into a congested nasal cavity or the narrow nasal cavity of a child. No prior steps are needed before collecting nasal secretions. The device enables rapid and convenient collection of nasal secretions and is applicable even for children.

We were able to obtain measurements for almost all samples, with the exception of 3 exceptionally viscous samples. The methodology used in this study is applicable to daily clinical practice. Another advantage of our method is the ability to measure allergen-specific IgE among the wide IgE repertoire offered by clinical laboratories. The CAP- immunoassay is the test designed and validated to measure allergen-specific IgE in the serum. Unlike the serum, nasal secretion contains many substances including IgA and mucin. These substances may interfere with the assay and may affect the results. Spike and recovery tests demonstrated that the assay was not affected

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the matrices of nasal secretions. Linearity of dilution tests proved the linearity of the assay. We believe that these two tests validated the accuracy of the assay in our study. Currently, possible clinical implications of measuring local IgE in nasal secretions include the measurement of specific IgEs from children, from whom collection of a blood sample can be difficult. Moreover, our methodology may contribute to further investigation of local IgE by providing a useful tool to obtain nasal secretions. However, the reproducibility and repeatability of our measurement method have not been confirmed, and additional fundamental studies are needed to validate the present approach.

The significance of local IgE in AR has yet to be investigated. Practical Guideline for the Management of Allergic Rhinitis in Japan[16] do not mention local IgE, and it is not currently used as an examination for AR.[17,18] Moreover, the Allergic Rhinitis and its Impact of Asthma (ARIA) criteria issued in 2008 refers to nasal-specific IgE and states that, based on current data, the concept of local allergic reaction in the nose without systemic IgE release is not fully supported and the measurement of IgE in nasal secretions cannot be routinely proposed. However, a growing number of articles have addressed local IgE in AR. A diagnostic flow-chart to detect forms of allergy different from the common IgE-mediated hypersensitivity, and incorporating local IgE, has been proposed.[19]

Some limitations must be considered when interpreting the present results. Measurement of allergen-specific IgE in nasal secretions and serum was carried out only once during the study period. In seasonal AR, levels of allergen-specific IgE in serum depend on the amount of allergen to which the patient has been exposed.[20,21] Our result might depict only part of the phenomenon, not the entire situation. Ideally, repeated measurement of allergen-specific IgE in both serum and nasal secretions over the year seems necessary to accurately evaluate the association between levels in these different compartments. Since the number of enrolled subjects was too small to reach a definitive conclusion, larger-scale studies are needed to confirm our result.

In conclusion, we measured JCP-specific IgE in the nasal secretions of subjects with and without Japanese cedar pollinosis using a novel methodology. A significantly high correlation was observed between levels of IgE in nasal secretions and in serum in symptomatic subjects. Although the current understanding of local IgE is insufficient and much remains to be studied, our data might offer some contributions and clinical implications regarding local IgE in AR.

## ACKNOWLEDGEMENTS

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