

Immunological parameters associated with the development of allergic rhinitis: A preliminary prospective study

AQ:1 Yasuhiro Uekusa,^{1,2} Ayako Inamine,¹ Syuji Yonekura,¹ Shigetoshi Horiguchi,¹ Takashi Fujimura,^{1,3} Daijyu Sakurai,¹ Heizaburo Yamamoto,¹ Homare Suzuki,¹ Toyoyuki Hanazawa,¹ and
 AQ:2 Yoshitaka Okamoto, M.D.,Ph.D.¹

ABSTRACT

Background: Many subjects are sensitized to Japanese cedar pollen but do not develop allergic rhinitis (AR). The aim of this study was to examine the immunologic parameters related to the development of AR in sensitized subjects.

Methods: The subjects were 33 adults who were sensitized to Japanese cedar pollen, but had not developed as of 2007. Cedar pollen-specific IgE (sIgE) and total IgE (tIgE) in serum, cedar pollen antigen (Cry j 1) Cry j-specific memory Th2 cell clone size, and the Cry j-specific induced regulatory T cell (iTreg) level were examined before and after the season in 2008.

Results: Eight of the 33 subjects developed cedar pollinosis. The sIgE titers before the season in these eight subjects did not differ from those in the subjects who did not develop pollinosis, but the titers after the season were significantly higher in the group that developed pollinosis. The sIgE/tIgE ratio increased in almost all subjects, but the ratio was significantly higher before the season in the subjects who developed pollinosis. Cry j-specific Th2 cells were detected in all subjects, but the clone size only increased in those that developed pollinosis. The Cry j-specific iTreg population did not differ between the two groups.

Conclusion: A high sIgE/tIgE ratio before the season may be predictive of development of pollinosis, and an increase in the allergen-specific Th2 clone size during the pollen season could be a biomarker for pollinosis. The role of allergen-specific iTreg cells in the development of pollinosis could not be clarified in this preliminary study.

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There has been a recent increase in the prevalence of allergic rhinitis (AR), with the prevalence rate exceeding 30% in many countries.^{1,2} Along with the burden of the disease and decrease in quality of life associated with AR, there are high costs related to the disorder.³⁻⁵ Genetic and environmental factors are involved in the onset of AR, as with many other diseases. The relevance of genetic factors can not be ignored, but environmental factors may have played a major role in the recent increase in allergic diseases.⁶⁻¹¹ Factors that change the predisposition to produce IgE are thought to have contributed to the recent increase of AR.

Many people have high sensitization rates to a variety of allergens, but some do not develop AR. A higher specific IgE (sIgE) titer is correlated with a higher incidence of AR,¹² but a significant number of subjects with a high sIgE titer do not have AR.^{13,14} The incidence rate also varies based on allergens, age, and gender.¹⁵⁻²¹ Subjects who are sensitized but do not develop AR might be thought of as being preconditioned to develop AR, and recent studies have also suggested that regulatory T cells (Treg) might play an important role in the development of allergic diseases.²²

Early intervention strategies are important for management of AR²³⁻²⁵ and clarification of the underlying mechanisms is required to develop an optimal strategy for secondary intervention. However, the mechanisms underlying development of AR are not well understood. In this preliminary study, we prospectively examined the immunologic parameters that may influence the development of AR.

MATERIALS AND METHODS

Inclusion Criteria

The study population consisted of subjects who complained of transient nasal symptoms and were diagnosed with an acute upper airway infection at our hospital in 2007. All subjects met the following inclusion criteria: a serum cedar pollen sIgE score of ≥ 2 on a CAP-radioallergosorbent test (CAP-RAST; SRL, Tokyo, Japan) performed at the end of 2007 (before the cedar pollen season in 2008), and no history of perennial AR or other pollinosis. Subjects with a history of bronchial asthma were also excluded from the study.

Diagnosis of Japanese Cedar Pollinosis

Diagnosis of cedar pollinosis was based on the following the criteria: symptoms of pollinosis, such as paroxysmal sneezing, runny nose, nasal congestion, nasal itching, and eye itching that persisted for >2 weeks during cedar pollen season (beginning in February and lasting until the middle of April), and positive identification of eosinophils in a nasal smear obtained during the peak of pollen dispersal. The symptoms and presence of eosinophils in the nasal smear disappeared after the pollen season.

Measurement of Parameters

Blood samples were collected before (December 2007) and after (April 2008) the cedar pollen season. Peripheral blood mononuclear cells (PBMCs) were obtained by the Ficoll-Hypaque method and stored in liquid nitrogen until analysis. Total IgE (tIgE) and sIgE titers for Japanese cedar, house-dust mites, and orchard grass were evaluated by the CAP-RAST method (Phadia).

Analysis of the Cry j-Specific Memory Th2 Clone Size

The number of IL-4-, IL-5-, or IL-13-producing cells after stimulation with Cry j 1 was determined by an enzyme-linked immunosorbent spot assay, as previously described.²⁶ Briefly, anti-human IL-4, IL-5, and IL-13 mAbs were diluted to a concentration of 15 $\mu\text{g}/\text{mL}$ in sterile, filtered (0.45 μm) PBS (pH 7.2), and added to nitrocellulose plates (Millititer; Millipore Corp., Bedford, MA) at 100 $\mu\text{L}/\text{well}$. The plates were incubated overnight at 4°C and unbound antibodies were

From the ¹Departments of Otolaryngology, Head and Neck Surgery and ²Immunology, Graduate School of Medicine, Chiba University, Chiba, Japan, and ³Reserch Center for Allergy and Immunology, Yokohama Institute, The Institute of Physical and Chemical Research (RIKEN), Yokohama, Kanagawa, Japan

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The authors have no conflicts of interest to declare pertaining to this article
 Address correspondence and reprint requests to Yoshitaka Okamoto, M.D., Ph.D., Department of Otolaryngology, Head and Neck Surgery, Graduate School of Medicine, Chiba University, 1-8-1, Inohana, Chuo-ku, Chiba 260-8670, Japan
 E-mail address: yokamoto@faculty.chiba-u.jp
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washed away with filtered PBS. After the last wash, the PBS was sucked through the membrane under a vacuum (Millipore Corp.). A prestimulated cell suspension (100 μ L) was added to each well in duplicate and the plates were incubated for 10 hours at 37°C. The cells were washed before addition of 100 μ L of biotinylated mAbs (1 μ g/mL) and incubation for 2 hours at room temperature. The plates were then washed and incubated for 90 minutes at room temperature with 100 μ L of streptavidin alkaline phosphatase (Mabtech, Stockholm, Sweden) at a dilution of 1:1000. The unbound conjugate was removed by another series of rinses before 100 μ L of BCIP/NBT substrate solution (BioRad, Richmond, CA) was added, and the plates were incubated at room temperature until dark spots emerged (1 hour). Color development was stopped by repeated rinsing with tap water. After drying, the spots were captured electronically and counted by computer analysis to avoid any visual bias, using an Auto Counter (ImmunoScan; CTL, Gmünd, Germany).

Flow Cytometric Analysis

The levels of Cry j 1-induced Treg (iTreg) and IL-10-Tr1 were analyzed by flow cytometry, as described previously.²⁷ Briefly, for intracellular staining of Foxp3 and IL-10, PBMCs were cultured with or without Cry j 1 for 3 days, followed by culture with 10 ng/mL of phorbol 12-myristate 13-acetate, 1 μ M of ionomycin, and 2 μ M of monensin for 6 hours. The PBMCs were stained with phycoerythrin-anti-CD25 (eBioscience, San Diego, CA) and phycoerythrin-Cy7-anti-CD4 (BD Biosciences, San Diego, CA) antibodies in PBS containing 1% FCS and 0.1% sodium azide for 20 minutes at 4°C. After surface staining, the PBMCs were stained with FITC-anti-Foxp3 (clone: PCH101; eBioscience) and allophycocyanin-anti-IL-10 (BD Biosciences) antibodies for 30 minutes at 4°C using a Foxp3 staining buffer set (eBioscience) according to the manufacturer's instructions.

To detect the Treg population, the first gate was set for mononuclear cells based on the profiles of their cell size, and then set a quadro gate to separate negative and positive populations for CD4 and CD25. The threshold of intensity for surface staining to separate the positive and negative populations was based on the intensity of the valley between the positive and negative peaks on each histogram plot for CD4 and CD25 staining. The threshold of intracellular staining was determined by considering the staining profiles of Cry j 1⁻ healthy subjects without antigen stimulation (negative controls). The intensity of staining control was confirmed to be weaker than the threshold of intracellular staining of the samples. The percentage of IL-10/Foxp3-double-positive cells and that of IL-10⁺ cells among the CD4/CD25-double-positive cells were calculated.

Pollen Counts

Cedar pollen dispersion in Chiba City was measured using a gravimetric method with a Durham sampler (Nishizaki Co., Ltd., Funabashi, Japan).

Statistical Analysis

Data were analyzed using two-tailed tests at a significance level of 5% or by chi-square test and Mann-Whitney *U* test.

Ethical Considerations

The study received prior approval from the Ethics Committee of Chiba University (Chiba, Japan). Written, witnessed, informed consent was obtained from all subjects.

RESULTS

Development of Japanese Cedar Pollinosis

The subjects were 33 adults (20 male and 13 female subjects) who were sensitized to Japanese cedar pollen but who had not developed cedar pollinosis as of December 2007. Age ranged from 18 to 41 years, with an average of 26.0 years. No subjects had a history of allergy (bronchial

Table 1 Baseline characteristics of the subjects

	AR ⁺	AR ⁻
Subjects	8	25
Sex (M/F)	4/4	16/9
Age, yr		
Mean	25.1 \pm 7.7	26.3 \pm 5.7
Range	18–41	19–37
sIgE titer (UA/mL)		
Mean	30.9 \pm 28.8	10.7 \pm 14.1
Range	2.62–79.5	0.77–57.7
tIgE titer (IU/mL)		
Mean	350.3 \pm 284.0	390.48 \pm 411.6
Range	54.7–886	7.3–1590
Sensitization to house-dust mites	6 (75%)	18 (72%)

sIgE and tIgE titers were measured at the end of 2007.

AR = allergic rhinitis; sIgE = cedar pollen-specific IgE; tIgE = total IgE.

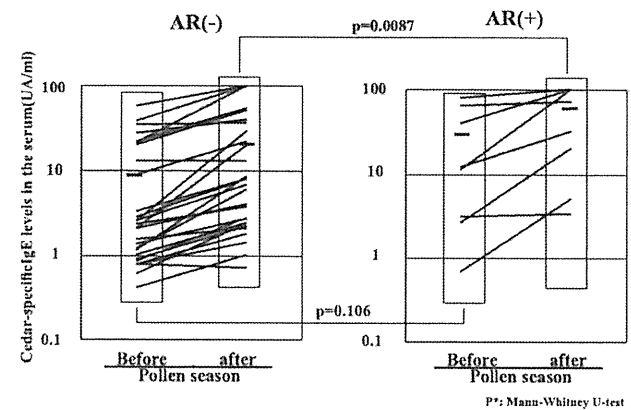


Figure 1. Cedar pollen-specific IgE levels in serum before and after pollen dispersal in 2008. The specific IgE (sIgE) titers before the pollen season did not differ significantly between subjects who did and did not develop pollinosis. During the pollen season, the levels increased in almost all subjects. The titers after the pollen season were significantly higher in subjects who developed pollinosis ($p = 0.0087$).

asthma, atopic dermatitis, or perennial AR), 25% had a family history of pollinosis, and 2% had a family history of bronchial asthma.

In 2008, cedar pollen dispersal started on February 20th and ended on April 10th, and the annual cedar pollen count was 4665/cm². Of the 33 subjects, 8 (4 male and 4 female subjects; 24%) developed cedar pollinosis in 2008. The ages of these 8 subjects ranged from 18 to 41 years (average, 25.1 years). In these subjects, cedar pollen sIgE titers before the cedar pollen season ranged from 2.62 to 79.5 UA/mL, tIgE titers ranged from 54.7 to 886 IU/mL, and 6 of the subjects (75%) were sensitized to dust mites. The 25 subjects (16 male and 9 female subjects) who did not develop pollinosis ranged in age from 19 to 37 years (average, 26.4 years), had cedar pollen sIgE titers before the cedar pollen season from 0.77 to 57.7 UA/mL, and tIgE titers from 7.3 to 1590 IU/mL, with 72% sensitized to dust mites. There were no significant differences in pollen-specific titers, tIgE titers, and sensitization to dust mites between the two groups (Table 1).

Cry j Pollen sIgE Levels in Serum

Serum sIgE levels are shown in Fig. 1. Cedar pollen sIgE titers before the pollen season did not differ significantly between subjects who did and did not develop pollinosis. Titers increased during the pollen season in almost all subjects, but after the pollen season the titers were significantly higher in subjects who developed pollinosis.

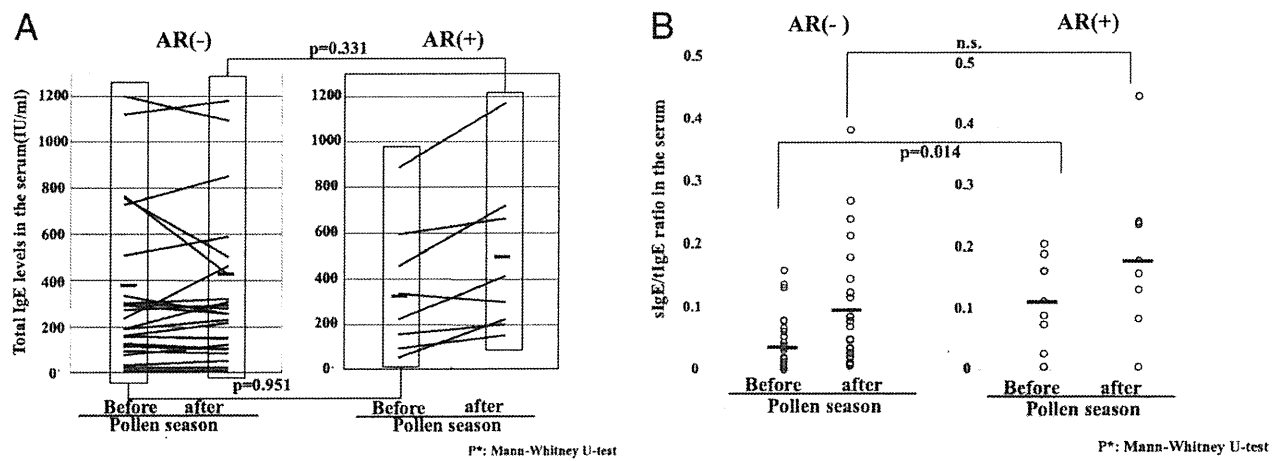


Figure 2. (A) Total IgE (IgE) levels in serum before and after pollen dispersal in 2008. The IgE titers did not differ significantly between subjects who did and did not develop pollinosis. During the pollen season, titers increased in almost all subjects and were not significantly different in the two groups. (B) The sIgE/IgE ratios before and after pollen dispersal in 2008. The average ratio of Japanese cedar pollen sIgE/IgE in serum before the pollen season was significantly higher in the AR⁺ group compared with the AR⁻ group (0.105 ± 0.074 versus 0.037 ± 0.042 ; $p = 0.022$).

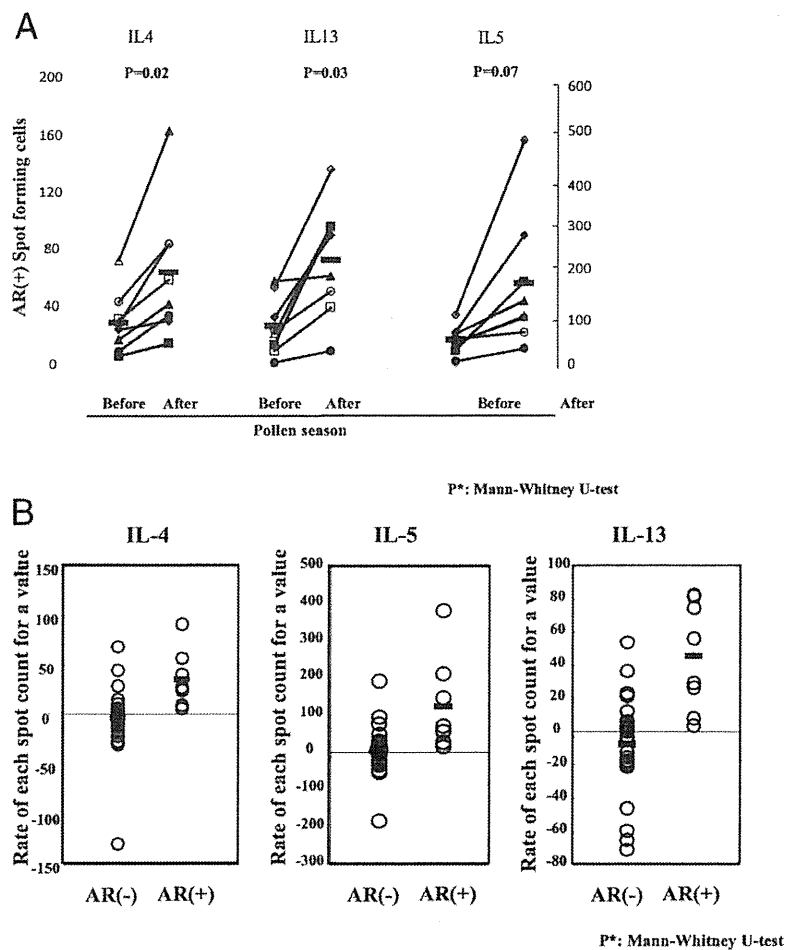


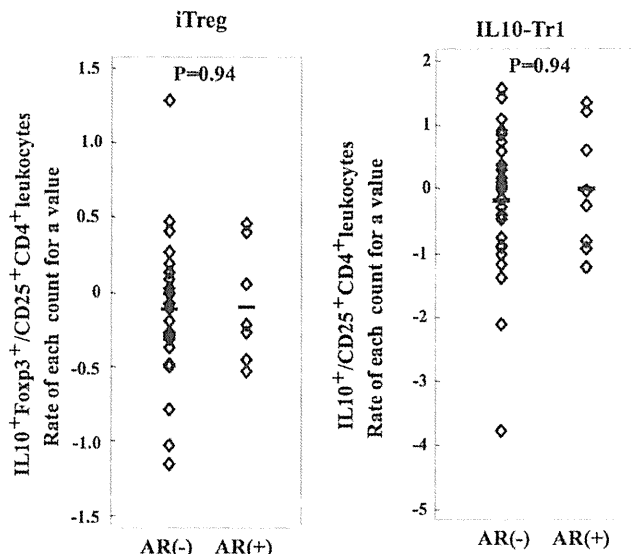
Figure 3. Cry j 1 peptide-specific IL-4-, IL-5-, and IL-13-producing cells were counted by enzyme-linked immunosorbent spot (ELISPOT) assay before and after the pollen season in 2008. Samples at each time point were analyzed simultaneously. (A) IL-4, IL-5, and IL-13 spots after the pollen season in subjects who did and did not develop cedar pollinosis, compared with those before the season ($p < 0.05$). (B) Relative change of Cry j-specific IL-4, IL-5, and IL-13 clone sizes after the pollen season, compared with those before the season ($p < 0.05$).

IgE Levels in Serum

F2 Total serum IgE levels are shown in Fig. 2 A. The IgE titers did not differ significantly between subjects who did and did not develop pollinosis. Titers increased during the pollen season in almost all subjects and were not significantly different in the two groups.

sIgE/IgE Ratio

The average ratio of Japanese cedar pollen sIgE to IgE in serum (sIgE/IgE) before the pollen season was significantly higher in subjects who developed pollinosis (0.105 ± 0.074 versus 0.037 ± 0.042 ; $p = 0.022$ by Mann-Whitney U test; Fig. 2 B).



P*: Mann-Whitney U-test

Figure 4. Detection of IL-10⁺ Foxp3⁺/CD25⁺ CD4⁺ T cells and IL-10⁺/CD25⁺CD4⁺ T cells in flow cytometric analysis of peripheral blood mononuclear cells (PBMCs). The relative changes from before to after the pollen season are shown for subjects who did and did not develop cedar pollinosis.

Cry j-Specific Memory Th2 Cell Clone Sizes

The numbers of antigen-specific IL-4, IL-5, and IL-13 spots are shown in Fig. 3 A. The number of IL-4 spots before the cedar pollen season was similar for subjects who did and did not develop pollinosis, but a significant increase in IL-4 spots after the pollen season occurred only for those that developed pollinosis. The same trend was obtained for IL-5 and IL-13 spots. Changes in Th2 clone sizes are shown in Fig. 3 B. The Th2 clone size in the group that developed pollinosis showed a significant increase after the pollen season.

Level of Cry j-Specific iTregs

We analyzed the population of IL-10⁺Foxp3⁺ cells and IL10⁺ cells in CD25⁺CD4⁺ leukocytes as potential markers for iTreg and IL10⁺Tr1 cells after stimulation with or without Cry j 1 before and after the pollen season in 2008. Flow cytometric analysis of the IL-10⁺Foxp3⁺/CD25⁺CD4⁺ T cells and IL-10⁺/CD25⁺CD4⁺ T cells among PBMCs is shown in Fig. 4. There were few Cry j-specific iTreg (0.2–1.9%) and Tr1 cells (1.1–2.9%) among the CD25⁺CD4⁺ T cells in all subjects before the pollen season. The number of Cry j-specific Tregs did not increase on pollen exposure and did not differ between the two groups of subjects or from before to after the cedar pollen season.

DISCUSSION

Eight of the 33 subjects who were sensitized to Japanese cedar pollen but who had not developed cedar pollinosis by the end 2007 developed cedar pollinosis in 2008. In this context, it is of note that cedar pollen counts in 2008 were higher than the average over the last 15 years (3000/cm³). In our prospective study, the serum sIgE titer increased in almost all subjects during the pollen season and was significantly higher after the pollen season in the group that developed pollinosis; however, no significant difference between the groups was observed before the pollen season. Subjects with a higher sIgE titer in serum are known to have a higher incidence of AR,^{11,13} but a high sIgE titer may not always predict development of AR. Thus, some subjects with a CAP-RAST score of 5 or 6 do not have AR.¹⁶

The sIgE/tIgE ratio in serum was significantly higher in subjects who developed pollinosis, even before the pollen season. In immunotherapy for grass pollinosis, the sIgE/tIgE ratio is significantly

higher in responders than in nonresponders.²⁸ However, in this study, a high sIgE/tIgE ratio was found to correlate with development of cedar pollinosis. This may reflect the amount of surface IgE on effector cells such as mast cells and basophils, and a low level of sIgE might cause these cells to be less likely to be activated by antigen cross-linking. Our results suggest that the sIgE/tIgE ratio is a more sensitive marker for prediction of onset of Japanese cedar pollinosis, compared with the serum sIgE titer, and this ratio might be a useful predictive marker for development of AR.

The profiles of allergen-specific Th cells differed between subjects who did and did not develop pollinosis. Cedar-specific IL-4-, IL-5-, and IL-13-producing memory T cells in peripheral blood were examined by enzyme-linked immunosorbent spot assay using Japanese cedar pollen-specific peptides. The number of cedar peptide-specific Th2 cells was low, but all subjects examined exhibited 5–100 spots/10⁵ of PBMCs. The size of the cedar pollen-specific Th2 cell clones did not differ between the two groups before the pollen season. However, the Cry j-specific Th2 clone sizes increased by 1.5-fold during the cedar pollen season in the group that developed pollinosis. This increase did not occur in subjects who did not develop pollinosis. The change in clone size may correlate with the allergen sIgE level and was more sensitive to this level compared with the change in serum sIgE as reported in our previous study.²⁶ The increased pollen-specific Th2 clone size is more susceptible to change and results in up-regulation of Th2-mediated immune responses by pollen exposure.

The immunologic mechanisms underlying the development of AR are complicated and depend on factors including the allergen, allergen exposure, age, and gender. However, elucidation of these mechanisms is necessary for promotion of early intervention. Recent studies have suggested the significance of Tregs^{21,29,30} and higher IL-10 levels in the off-season in AR patients without bronchial hyperresponsiveness, compared with those with bronchial hyperresponsiveness.³¹ In our study, there were few Tregs in subjects who were sensitized to Japanese cedar pollen but who had not developed pollinosis, and these Tregs did not increase after pollen exposure. The suppression of allergen-specific Th2 clones observed in this study may have been induced through development of Tregs, although no significant contribution of specific iTreg and IL-10⁺ Tr1 cells was observed. Therefore, the precise composition of the various types of T cells requires clarification in further studies.

In summary, this preliminary study suggests that development of cedar pollinosis is associated with a high sIgE/tIgE ratio before pollen exposure and with an increased specific Th2 lymphocyte clone size induced by exposure to cedar pollen. The precise role of the sIgE/tIgE ratio and the contribution of T-cell subsets need to be examined in a large cohort study.

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