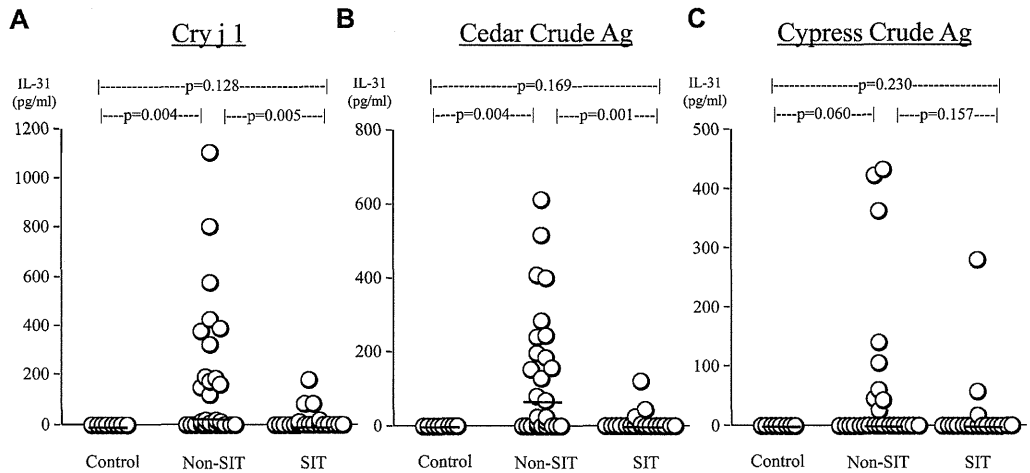


FIG 1. Production of IL-31 by PBMCs after stimulation with pollen antigens. PBMCs from control subjects *(Control)*, patients with JCCP without specific immunotherapy *(Non-SIT)*, and patients with JCCP with specific immunotherapy *(SIT)* were stimulated with Cry j 1 **(A)**, cedar crude antigen **(B)**, and cypress crude antigen **(C)**, and then the concentration of IL-31 in the supernatants was measured. The *horizontal line* indicates the median. *P* values were determined by using the Mann-Whitney *U* test. *Ag*, Antigen.

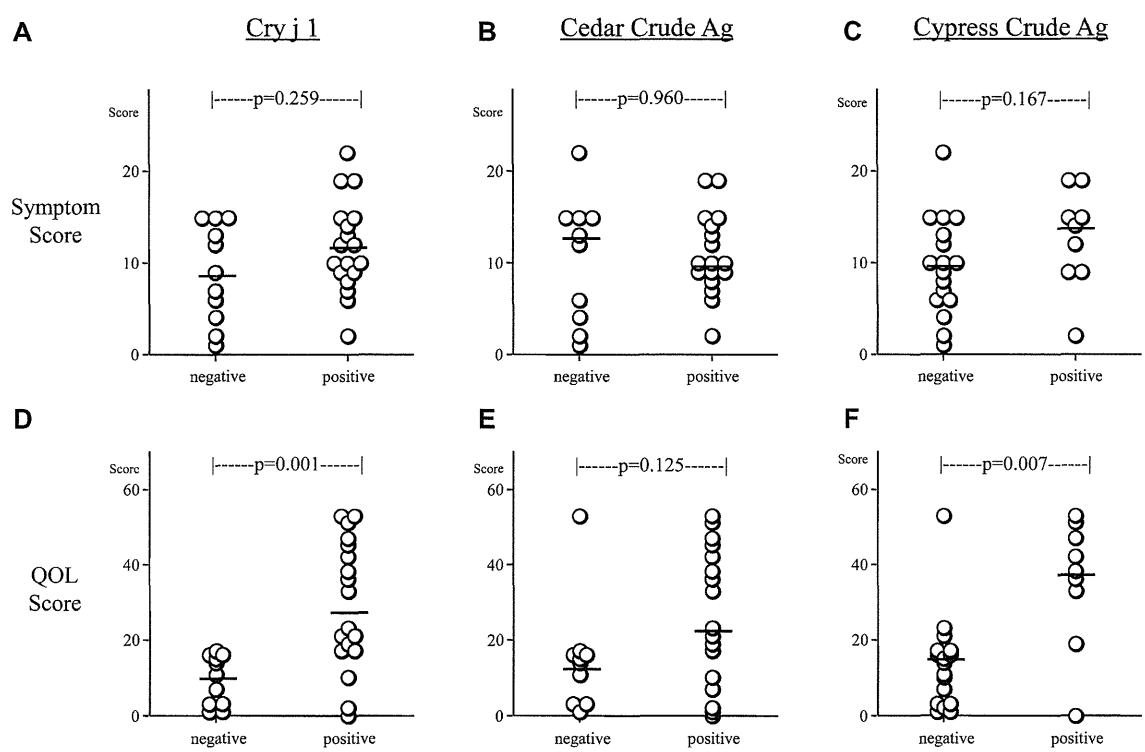


FIG 2. Comparison of naso-ocular symptoms **(A-C)** and QOL **(D-F)** during the peak season of cypress pollen dispersion between patients with positive and negative production of IL-31 in response to Cry j 1 (Fig 2, *A* and *D*), cedar crude antigen (Fig 2, *B* and *E*), and cypress crude antigen (Fig 2, *C* and *F*). The *horizontal line* indicates the median. *P* values were determined by using the Mann-Whitney *U* test. *Ag*, Antigen.

org). The levels of IFN-γ production were not different between IL-31 producers and nonproducers, except for cedar crude antigen stimulation (see Fig E2, *C*, *F*, and *I*). These results suggest that antigen-induced IL-31 production is selectively associated with T_H2 responses in PBMCs.

Symptom scores in the peak season of cedar pollen dispersion were similar between IL-31 producers and nonproducers in response to Cry j 1, cedar crude antigen, and cypress crude antigen (see Fig E3, *A-C*, in this article's Online Repository at www.jacionline.org). QOL scores in the peak season of cedar pollen dispersion were also similar between IL-31 producers and nonproducers in response to cedar crude antigen and cypress crude antigen (see Fig E3, *E* and *F*). A trend in the exacerbation of QOL was seen in Cry j 1–induced IL-31 producers compared with nonproducers; however, this trend did not reach statistical difference ($P = .087$; see Fig E3, *D*).

Symptom scores in the peak season of cypress pollen dispersion were also similar between IL-31 producers and nonproducers in response to Cry j 1, cedar crude antigen, and cypress crude antigen (Fig 2, *A-C*). However, QOL scores, in which a high score means a low QOL, in the peak season of cypress pollen dispersion were significantly higher in IL-31 producers in response to Cry j 1 and cypress crude antigen, but not cedar crude antigen, compared with those seen in the respective nonproducers (Fig 2, *D-F*). Together with the finding that PBMCs that produced IL-31 in response to pollen antigens produced higher amounts of IL-5 and IL-13 in response to the respective antigens, these results suggest that the induction of IL-31 production might lead to a deterioration of JCCP.

The amount of IL-31 production in response to pollen antigens did not correlate with symptom or QOL scores in the peak season of cedar pollen dispersion (see Fig E4 in this article's Online Repository at www.jacionline.org). However, the amounts, especially in response to Cry j 1 ($\rho = 0.641$, $P < .001$) and cypress crude antigen ($\rho = 0.658$, $P = .002$), significantly and positively correlated with QOL scores in the peak season of cypress pollen dispersion (see Fig E5, *D-F*, in this article's Online Repository at www.jacionline.org). In addition, there was a trend for a positive correlation between cypress crude antigen–induced IL-31 production and symptom scores in the season ($\rho = 0.451$, $P = .070$; see Fig E5, *C*). In contrast, the amounts of IL-5 or IL-13 production after stimulation with pollen antigens did not correlate with the QOL scores (see Fig E6 in this article's Online Repository at www.jacionline.org). This result suggests that the induction of pollen antigen–induced IL-31 production by PBMCs is associated with the severity of allergic rhinitis. Detailed discussion is available in this article's Discussion section and Figs E8 and E9 in this article's Online Repository at www.jacionline.org.

The present study provides evidence that, unlike other T_H2-type cytokines, including IL-5 and IL-13, IL-31 displays a unique and independent role in the pathophysiology of allergic rhinitis. The amount of pollen antigen–induced IL-31 production by PBMCs is selectively associated with the severity of QOL in patients with JCCP. These observations might provide a basis for future therapeutic approaches targeting IL-31 in the management and alleviation of allergic rhinitis.

Mitsuhiro Okano, MD[a]
Tazuko Fujiwara, BS[a]
Takaya Higaki, MD[a]
Seiichiro Makihara, MD[a]
Takenori Haruna, MD[a]
Yohei Noda, MD[a]
Kengo Kanai, MD[a]
Shin Kariya, MD[a]
Hiroshi Yasueda, PhD[b]
Kazunori Nishizaki, MD[a]

From [a]the Department of Otolaryngology–Head & Neck Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan, and [b]the Clinical Research Center for Allergy and Rheumatology, Sagamihara National Hospital, Sagamihara, Japan. E-mail: mokano@cc.okayama-u.ac.jp.

Supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology, Japan (20592001).

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

REFERENCES

1. Okubo K, Goto M, Fujieda S, Okano M, Yoshida H, Morikawa H, et al. A randomized-double-blind comparative study of sublingual immunotherapy for cedar pollinosis. Allergol Int 2008;57:265-7.
2. Sone K, Dairiki K, Morikubo K, Shimizu K, Tsunoo H, Mori T, et al. Recognition of T cell epitopes unique to Cha o 2, the major allergen in Japanese cypress pollen, in allergic patients cross-reactive to Japanese cedar and Japanese cypress pollen. Allergol Int 2009;58:237-45.
3. Dillon SR, Sprecher C, Hammond A, Bilsborough J, Rosenfeld-Franklin M, Presnell SR, et al. Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. Nat Immunol 2004;5:752-60.
4. Bilsborough J, Leung DYM, Maurer M, Howell M, Boguniewcz M, Yao L, et al. IL-31 is associated with cutaneous lymphocyte antigen-positive skin homing T cells in patients with atopic dermatitis. J Allergy Clin Immunol 2006;117:418-25.
5. Neis MM, Peters B, Dreuw A, Wenzel J, Bieber T, Mauch C, et al. Enhanced expression levels of IL-31 correlate with IL-4 and IL-13 in atopic and allergic contact dermatitis. J Allergy Clin Immunol 2006;118:930-7.
6. Raap U, Wichmann K, Bruder M, Stander S, Wedi B, Kapp A, et al. Correlation of IL-31 serum levels with severity of atopic dermatitis. J Allergy Clin Immunol 2008; 122:421-3.
7. Perrigoue JG, Li J, Zaph C, Goldschmidt M, Scott P, de Sauvage FJ, et al. IL-31-IL-31R interactions negatively regulate type 2 inflammation in the lung. J Exp Med 2007;204:481-7.
8. Ip WK, Wong CK, Li MLY, Li PW, Cheung PFY, Lam CWK. Interleukin-31 induces cytokine and chemokine production from human bronchial epithelial cells through activation of mitogen-activated protein kinase signaling pathways: implications for the allergic diseases. Immunology 2007;122:532-41.
9. Lei Z, Liu G, Huang Q, Lv M, Zu R, Zhang GM, et al. SCF and IL-31 rather than IL-17 and BAFF are potential indicators in patients with allergic asthma. Allergy 2007;63:327-32.

doi:10.1016/j.jaci.2010.09.029

Sequence variation in the *IL4* gene and resistance to *Trypanosoma cruzi* infection in Bolivians

To the Editor:

Chagas disease, caused by the parasite *Trypanosoma cruzi*, affects 10 to 12 million people each year in Latin America, with Bolivia having the highest prevalence of infection (see "Outlook: Chagas disease"[1] and references therein). In the chronic phase, Chagas infection may present as an indeterminate form in which 60% of infected individuals remain asymptomatic despite having positive serologic reactions for *T cruzi*. In the remaining 40% of patients with Chagas disease, tissue inflammation leads to organ damage, affecting the cardiac, digestive, or nervous systems up to 25 years after initial infection. Several studies identified genetic markers for disease establishment and progression in Venezuelans, Brazilians, Peruvians, Colombians, and Mexicans,[2] but no genetic studies have been conducted previously in Bolivians.

Cytokines produced in response to *T cruzi* infection appear to modulate disease progression by enhancing or inhibiting parasite replication in a variety of cell types. In particular, the T_H2 cytokine IL-4 maintains inflammation and parasite persistence in Chagas disease,[3] whereas T_H1 cytokines maintain control of parasitism[4] but can also contribute to the development of chronic myocarditis.[5]

To determine whether genetic variation at the *IL4* gene is associated with *T cruzi* infection in Bolivians, we performed a resequencing study of an approximately 12-kb region around the *IL4* locus, including 470 base pairs (bp) of coding (exon) sequence, 368 bp of 5′ untranslated region, 82 bp of 3′ untranslated region, and 11,453 bp of intronic sequence. The study included 110 individuals from the Department of Cochabamba, Bolivia, with infection status serologically confirmed by 2 different diagnostic tests (HAI Chagas Polychaco; Laboratorio Lemos, S.R.L., Buenos Aires, Argentina, and IFI Biocientifica S.A., Buenos Aires, Argentina). Each subject was classified according to the serologic results as a case (positive serology) or a control (negative

METHODS

Antigens and reagents

Crude antigens of Japanese cedar pollen and Japanese cypress pollen were extracted from *Cryptomeria japonica* pollen and *Chamaecyparis obtuse* pollen, respectively, as described previously.[E1,E2] Cry j 1 was purified and concentrated from Japanese cedar crude antigen, as previously described.[E1]

Patients

Forty-nine patients with JCCP (15 men and 34 women; age range, 29-75 years; mean age, 51.0 years) were enrolled in the study. Written informed consent was obtained from each subject. Sensitization to Japanese cedar pollen was confirmed by the presence of specific IgE antibodies (range, 0.73 to >100 UA/mL; mean, 20.54 ± 22.39 UA/mL), as determined by means of ImmunoCAP (Phadia AB, Uppsala, Sweden). Twenty patients received SIT with a standardized extract of *C japonica* pollen (Torii Co, Tokyo, Japan) over a period of at least 2 years. The mean maintenance dose of the extract was 468 JAU. None of the patients had used immunosuppressive drugs, including oral steroids, during the pollen season. The control group consisted of 8 healthy subjects with no sensitization to Japanese cedar pollen, as confirmed by means of ImmunoCAP (3 men and 5 women; age range, 34-62 years; mean age, 46.5 years). No significant differences in age or sex existed among the 3 groups. The study was approved by the Human Research Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences.

Antigen-specific cytokine production by PBMCs

Heparinized blood was collected from May to June 2009. PBMCs were isolated and cultured as previously described.[E3] In brief, PBMCs (2×10^6/mL) were incubated in the presence or absence of 10 μg/mL cedar crude antigen, cypress crude antigen, or Cry j 1 at 37°C in a 5% CO_2/air mixture for 72 hours. PBMCs from all 29 patients with JCCP who were not treated with SIT were examined for cytokine production in response to Cry j 1. However, because of limited sample volumes, PBMCs from 27 and 26 patients with JCCP not treated with SIT were examined for cytokine production in response to cedar crude antigen and cypress crude antigen, respectively. All PBMCs from SIT-treated patients could be examined for cytokine production in response to the 3 antigens. Then supernatant was collected and stored at −80°C until it was assayed. Levels of IL-5, IL-13, and IFN-γ were measured by using Opt EIA sets (BD Biosciences, San Jose, Calif) in accordance with the manufacturer's instructions. Levels of IL-31 were measured by using a DuoSet ELISA development kit (R&D Systems, Minneapolis, Minn). The detection limit of these assays was 3.9 pg/mL for IL-5, 3.9 pg/mL for IL-13, 7.8 pg/mL for IFN-γ, and 7.8 pg/mL for IL-31.

Monitoring of symptoms and QOL

Japanese rhinoconjunctivitis QOL questionnaires were used to compare naso-ocular symptoms and rhinitis-related QOL during the pollen dispersion season between SIT-treated and SIT-untreated patients.[E4]

A total of 2,220 grains/cm^2 of Japanese cedar pollen and 1,478 grains/cm^2 of Japanese cypress pollen were dispersed in 2009. The peak dispersions of cedar and cypress pollens occurred on March 10 and April 11, respectively (Fig E7). Thus patients answered the Japanese rhinoconjunctivitis QOL questionnaires during March 4 to 18 and April 8 to 22 to assess naso-ocular symptoms and rhinitis-related QOL.

Statistical analysis

Median values are presented. The nonparametric Mann-Whitney *U* test was used to detect differences between groups. Correlation analysis was performed by using the Spearman correlation coefficient by rank. *P* values of less than .05 were considered statistically significant. The statistical analysis was performed with StatView software (version 4.5; Abacus, Inc, Berkeley, Calif).

DISCUSSION

In the present study we characterized the IL-31 production in pollen antigen–induced PBMC responses in patients with allergic rhinitis. Evidence is accumulating regarding the role of IL-31 in the pathogenesis of allergic diseases, especially atopic dermatitis; however, this is the first report to demonstrate the role of IL-31 in patients with allergic rhinitis.[E5-E11]

The amount of pollen antigen–induced IL-31 production was significantly and positively correlated with the production of IL-5 and IL-13 but not IFN-γ. This result is consistent with the report by Neis et al[E7] that the expression of IL-31 mRNA in the skin was correlated with the expression of IL-4 and IL-13 in patients with atopic dermatitis. Although IL-31 can be produced by mast cells, this result further suggests that the main producer of IL-31 might be T_H2 cells producing both IL-5 and IL-13.[E12]

The most important and interesting finding in the present study is that the PBMCs from some patients with JCCP produced IL-31 in response to antigen, whereas the PBMCs from other patients with JCCP did not. About one third of the patients did not produce IL-31 in response to cedar pollen–related antigens (Cry j 1 and cedar crude antigen). On the other hand, most patients produced other T_H2 cytokines, IL-5, and IL-13 (Fig E8). In particular, all patients with JCCP produced IL-5 in response to Cry j 1 and cedar crude antigen, whereas the PBMCs from healthy control subjects did not produce IL-5. One of the reasons why all patients with JCCP produced IL-5 in response to the pollen antigens is the high pollen dispersion in 2009. This result is consistent with previous reports that the induction of antigen-specific IL-5 production by PBMCs was a key factor in the onset of allergic rhinitis, including JCCP.[E13,E14] In addition, PBMCs from about two thirds of the patients with JCCP did not respond to cypress crude antigen. It has been proposed that a subset of patients with atopic dermatitis express low levels of IL-31.[E6,E7] For example, cutaneous lymphocyte–associated antigen–positive T cells from 5 of 12 patients with atopic dermatitis did not produce IL-31 in response to a suboptimal concentration of anti-CD3.[E6] Our results are similar to these reports and suggest that patients with JCCP can be divided into 2 subsets regarding antigen-induced IL-31 production by PBMCs. In addition, these results suggest that the induction of IL-31 production is less essential for the onset of allergic rhinitis compared with other T_H2 cytokines, especially IL-5.

On the other hand, PBMCs that produced IL-31 in response to pollen antigens produced higher amounts of IL-5 and IL-13 in response to the respective antigens. This result is similar to the recent report by Woodruff et al[E15] that airway gene expression in patients with asthma can be divided into 2 distinct "T_H2-high" and "T_H2-low" subgroups. In addition, patients whose PBMCs produced IL-31 in response to Cry j 1 and cypress crude antigen had significantly impaired QOL at the peak season of cypress pollen dispersion. This result suggests that the induction of IL-31 production might lead to a deterioration in the pathophysiology of JCCP.

Furthermore, the amount of IL-31 produced by PBMCs in response to Cry j 1 and cypress crude antigen significantly and positively correlated with QOL scores in the peak season of cypress pollen dispersion. In human subjects with atopic dermatitis, IL-31 serum levels were correlated with the severity of atopic dermatitis, as determined by SCORAD scores.[E8] On the other hand, SCORAD scores were not correlated with the cutaneous expression levels of IL-31 mRNA.[E7] Our result is consistent with the former report and suggests that the induction of pollen

antigen–induced IL-31 production by PBMCs is associated with the severity of allergic rhinitis.

In the present study we used Cry j 1, the major allergen molecule of Japanese cedar pollen, as a purified pollen allergen. Cross-allergenicity between Cry j 1 and Cha o 1, the major allergen molecule of Japanese cypress pollen, has been reported at the human T-cell level.[E16,E17] In addition, we found that the amounts of IL-5 produced by PBMCs in response to Cry j 1 are significantly and highly correlated with those in response to Cha o 1 ($n = 46$, $r = 0.952$, $P < .001$, Pearson correlation coefficient, unpublished data, Fig E9). Thus the correlation between QOL scores in the peak season of cypress pollen dispersion and the amounts of IL-31 by PBMCs in response to Cry j 1 is due to the cross-reactivity of Cry j 1 and Cha o 1 at the cellular level.

The amount of pollen-induced IL-31 produced by PBMCs was correlated with QOL scores in the peak season of cypress pollen dispersion but not the peak season of cedar pollen dispersion. This might be due to the time of blood sampling. We collected blood from May to June 2009, just after the cessation of cypress pollen dispersion. Thus the amounts of cytokines produced by PBMCs might more closely reflect the pathogenesis caused by cypress pollen.

REFERENCES

E1. Yasueda H, Yui Y, Shimizu T, Shida T. Isolation and partial characterization of the major allergen from Japanese cedar (*Cryptomeria japonica*) pollen. J Allergy Clin Immunol 1983;71:77-86.

E2. Suzuki M, Komiyama N, Itoh M, Itho H, Sone T, Kino K, et al. Purification, characterization and molecular cloning of Cha o 1, the major allergen of *Chamaecyparis obtuse* (Japanese cypress) pollen. Mol Immunol 1996;33:451-60.

E3. Okano M, Otsuki N, Azuma M, Fujiwara T, Kariya S, Sugata Y, et al. Allergen-specific immunotherapy alters the expression of BTLA, a co-inhibitory molecule, in allergic rhinitis. Clin Exp Allergy 2008;38:1891-900.

E4. Okuda M, Ohkubo K, Goto M, Okamoto H, Konno A, Baba K, et al. Comparative study of two Japanese rhinoconjunctivitis quality-of-life questionnaires. Acta Otolaryngol 2005;125:736-44.

E5. Dillon SR, Sprecher C, Hammond A, Bilsborough J, Rosenfeld-Franklin M, Presnell SR, et al. Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. Nat Immunol 2004;5:752-60.

E6. Bilsborough J, Leung DYM, Maurer M, Howell M, Boguniewcz M, Yao L, et al. IL-31 is associated with cutaneous lymphocyte antigen-positive skin homing T cells in patients with atopic dermatitis. J Allergy Clin Immunol 2006;117:418-25.

E7. Neis MM, Peters B, Dreuw A, Wenzel J, Bieber T, Mauch C, et al. Enhanced expression levels of IL-31 correlate with IL-4 and IL-13 in atopic and allergic contact dermatitis. J Allergy Clin Immunol 2006;118:930-7.

E8. Raap U, Wichmann K, Bruder M, Stander S, Wedi B, Kapp A, et al. Correlation of IL-31 serum levels with severity of atopic dermatitis. J Allergy Clin Immunol 2008;122:421-3.

E9. Perrigoue JG, Li J, Zaph C, Goldschmidt M, Scott P, de Sauvage FJ, et al. IL-31-IL-31R interactions negatively regulate type 2 inflammation in the lung. J Exp Med 2007;204:481-7.

E10. Ip WK, Wong CK, Li MLY, Li PW, Cheung PFY, Lam CWK. Interleukin-31 induces cytokine and chemokine production from human bronchial epithelial cells through activation of mitogen-activated protein kinase signaling pathways: implications for the allergic diseases. Immunology 2007;122:532-41.

E11. Lei Z, Liu G, Huang Q, Lv M, Zu R, Zhang GM, et al. SCF and IL-31 rather than IL-17 and BAFF are potential indicators in patients with allergic asthma. Allergy 2007;63:327-32.

E12. Niyonsaba F, Ushio H, Hara M, Yokoi H, Tominaga N, Takamori K, et al. Antimicrobial peptides human beta-defensins and cathelicidin LL-37 induce the secretion of a pruritogenic cytokine IL-31 by human mast cells. J Immunol 2010;184:3526-34.

E13. Ohashi Y, Nakai Y, Tanaka A, Kakinoki Y, Masamoto T, Kato A, et al. Allergen-induced synthesis of interleukin-5, but not of IgE, is a key mechanism linked to symptomatic episodes of seasonal allergic rhinitis in sensitized individuals. Scand J Immunol 1998;47:596-602.

E14. Sun J, Wong B, Cundall M, Goncharova S, Conway M, Dalrymle A, et al. Immunoreactivity profile of peripheral blood mononuclear cells from patients with ragweed-induced allergic rhinitis. Clin Exp Allergy 2007;37:901-8.

E15. Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, et al. T-helper type 2-driven inflammation defines major subphenotypes of asthma. Am J Respir Crit Care Med 2009;180:388-95.

E16. Sone K, Dairiki K, Morikubo K, Shimizu K, Tsunoo H, Mori T, et al. Identification of human T cell epitopes in Japanese cypress pollen allergen, Cha o 1, elucidates the intrinsic mechanism of cross-allergenicity between Cha o 1 and Cry j 1, the major allergen of Japanese cedar pollen, at the T cell level. Clin Exp Allergy 2005;35:664-71.

E17. Nakamura Y, Takagi S, Suzuki M, Ito H, Murakami S, Ohta N. Survival of memory T cells specific for Japanese cypress pollen allergen is maintained by cross-stimulation of putative pectase lyase from other plants. Allergy 2001;56:285-92.

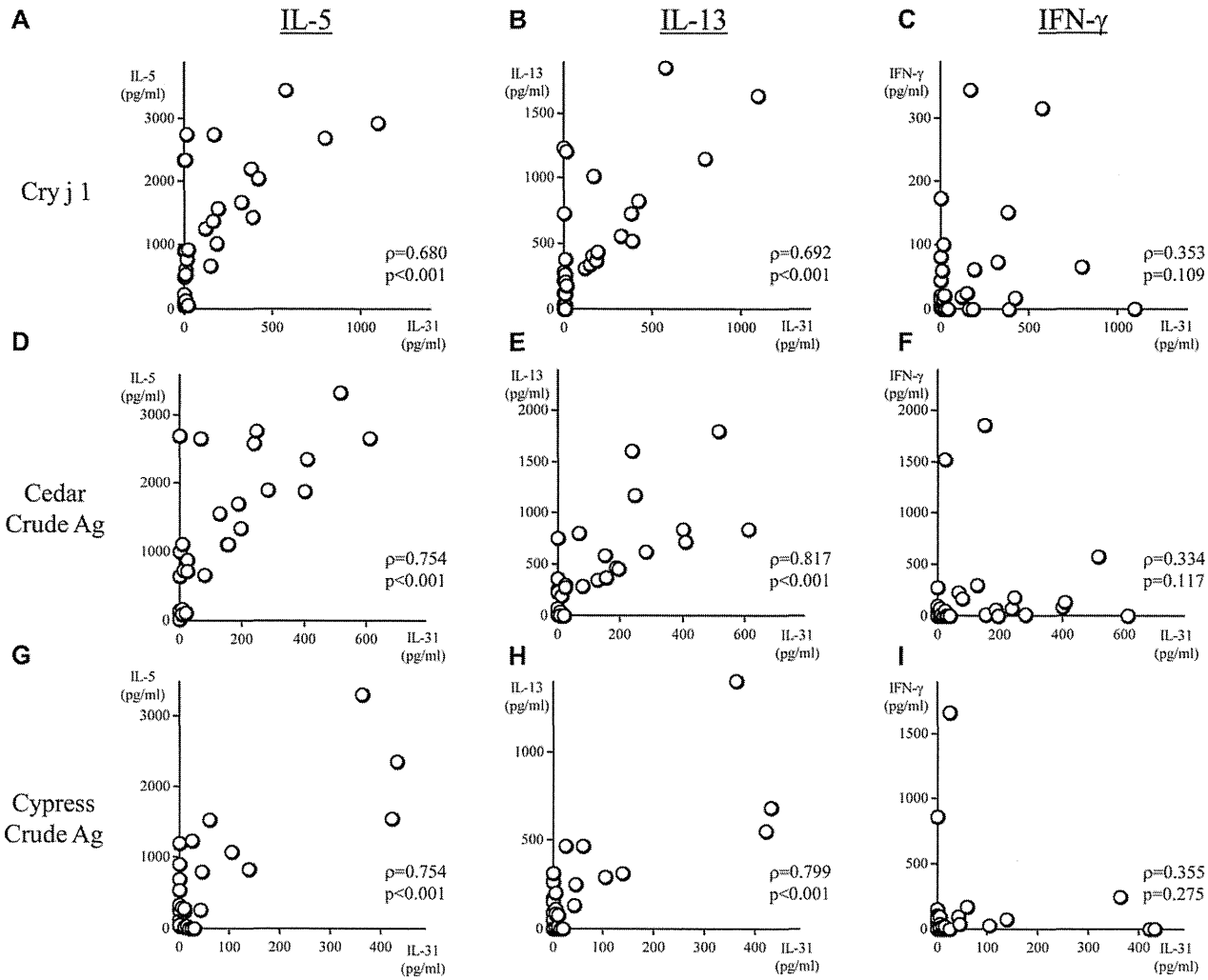


FIG E1. Relationship between amounts of pollen antigen–induced IL-31 and T_H1/T_H2 cytokines produced by PBMCs from patients with JCCP. Correlations between the amounts of IL-31 in response to Cry j 1 **(A-C)**, cedar crude antigen **(D-F)**, and cypress crude antigen **(G-I)** and the amounts of IL-5 (Fig E1, *A*, *D*, and *G*), IL-13 (Fig E1, *B*, *E*, and *H*), and IFN-γ (Fig E1, *C*, *F*, and *I*) in response to the respective antigens were determined by using the Spearman correlation coefficient by rank. *Ag*, Antigen.

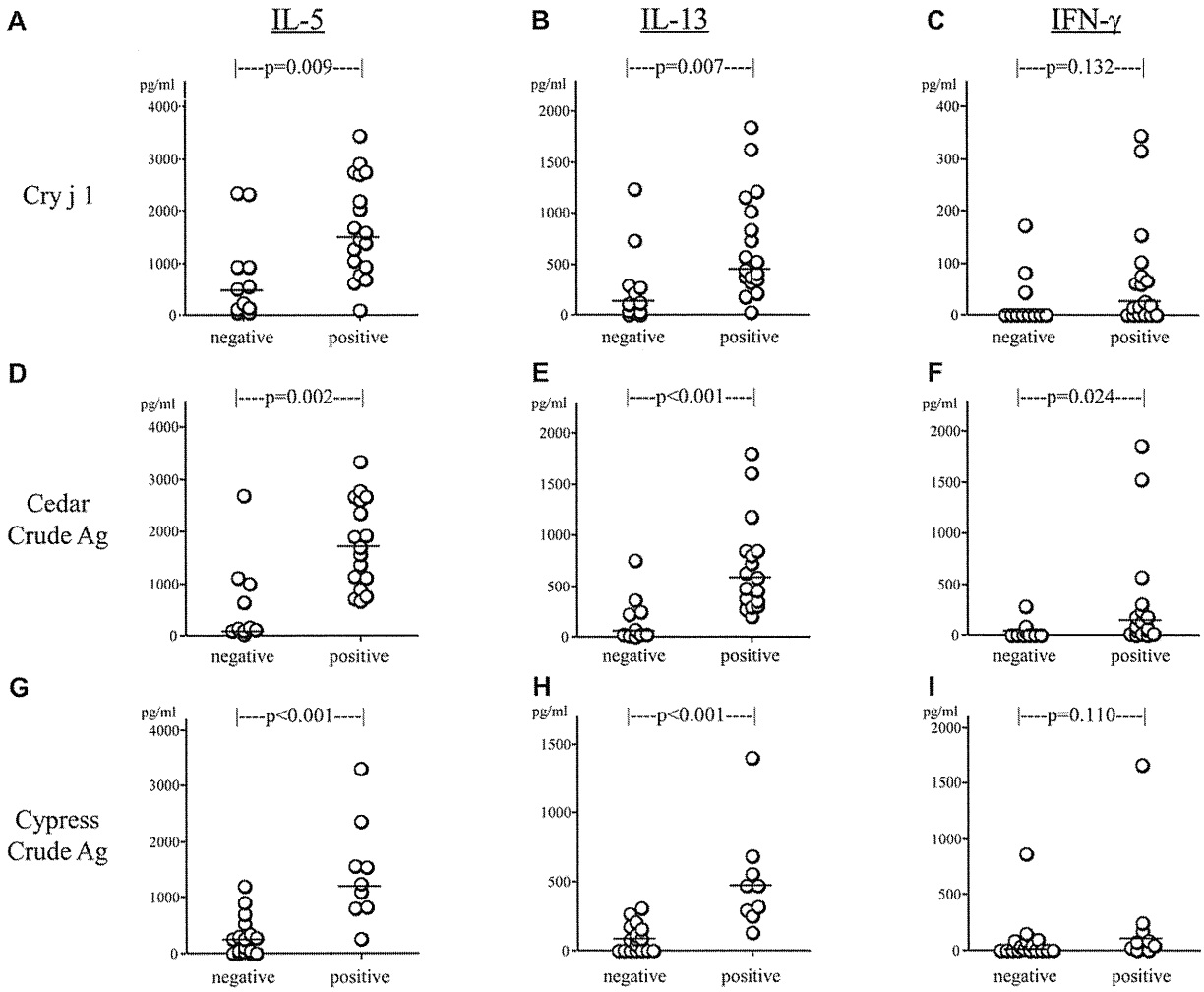


FIG E2. Comparison of the amounts of IL-5 (**A**, **D**, and **G**), IL-13 (**B**, **E**, and **H**), and IFN-γ (**C**, **F**, and **I**) in response to Cry j 1 (Fig E2, *A-C*), cedar crude antigen (Fig E2, *D-F*), and cypress crude antigen (Fig E2, *G-I*) between patients with positive and negative production of IL-31 in response to the respective antigens. The *horizontal line* indicates the median. *P* values were determined by using the Mann-Whitney *U* test. *Ag*, Antigen.

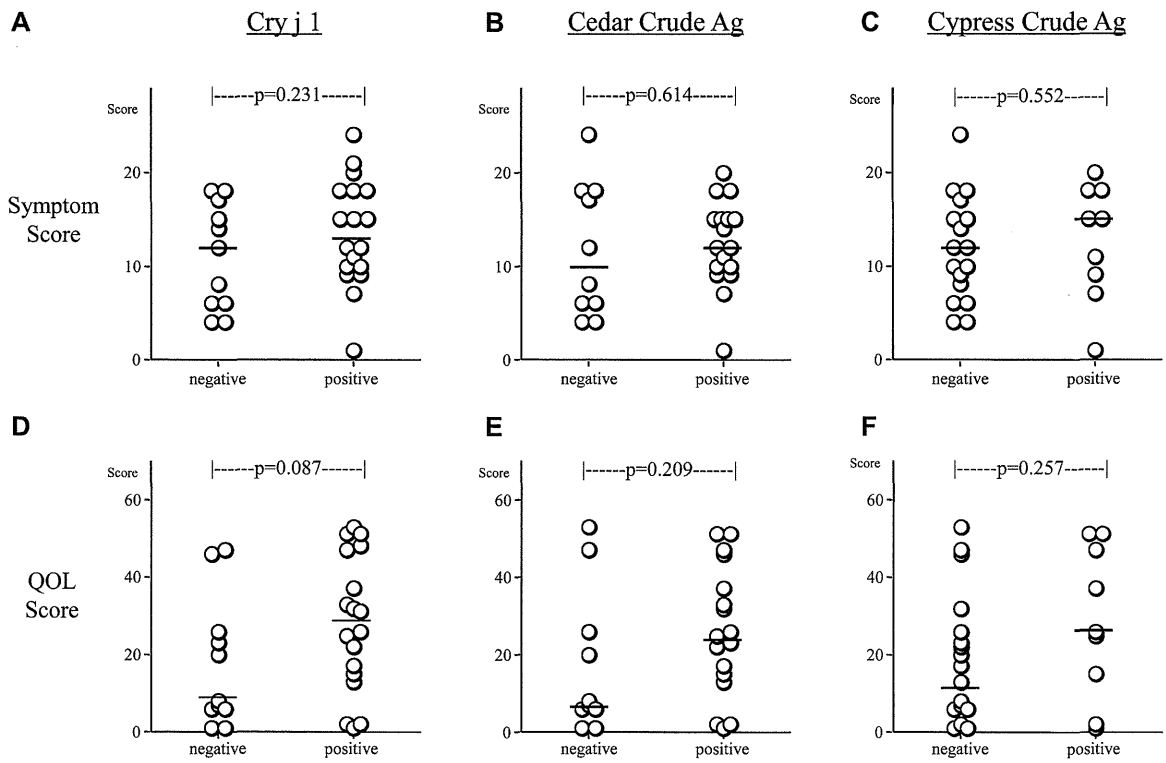


FIG E3. Comparison of naso-ocular symptoms **(A-C)** and QOL **(D-F)** during the peak season of cedar pollen dispersion between patients with positive and negative production of IL-31 in response to Cry j 1 (Fig E3, *A* and *D*), cedar crude antigen (Fig E3, *B* and *E*), and cypress crude antigen (Fig E3, *C* and *F*). The *horizontal line* indicates the median. *P* values were determined by using the Mann-Whitney *U* test. *Ag*, Antigen.

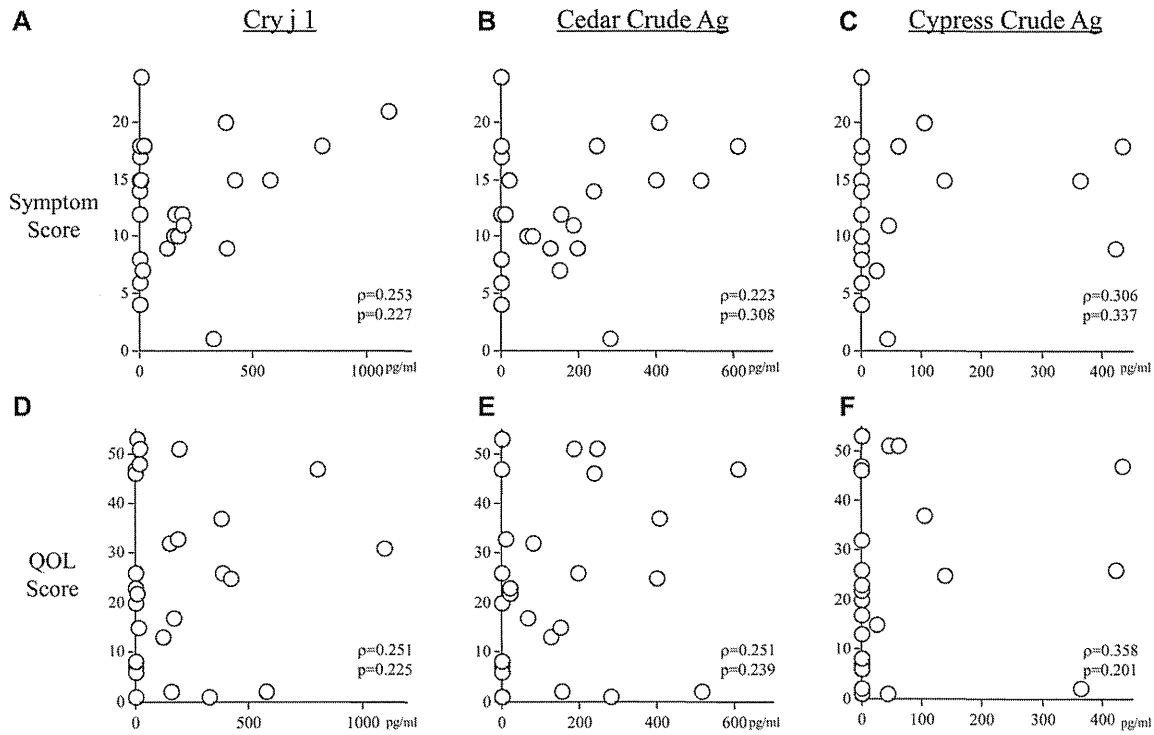


FIG E4. Relationship between naso-ocular symptoms **(A-C)** and QOL **(D-F)** during the peak season of cedar pollen dispersion and the amounts of IL-31 produced by PBMCs in response to Cry j 1 (Fig E4, *A* and *D*), cedar crude antigen (Fig E4, *B* and *E*), and cypress crude antigen (Fig E4, *C* and *F*). The ρ and *P* values were determined by using the Spearman correlation coefficient by rank. *Ag*, Antigen.

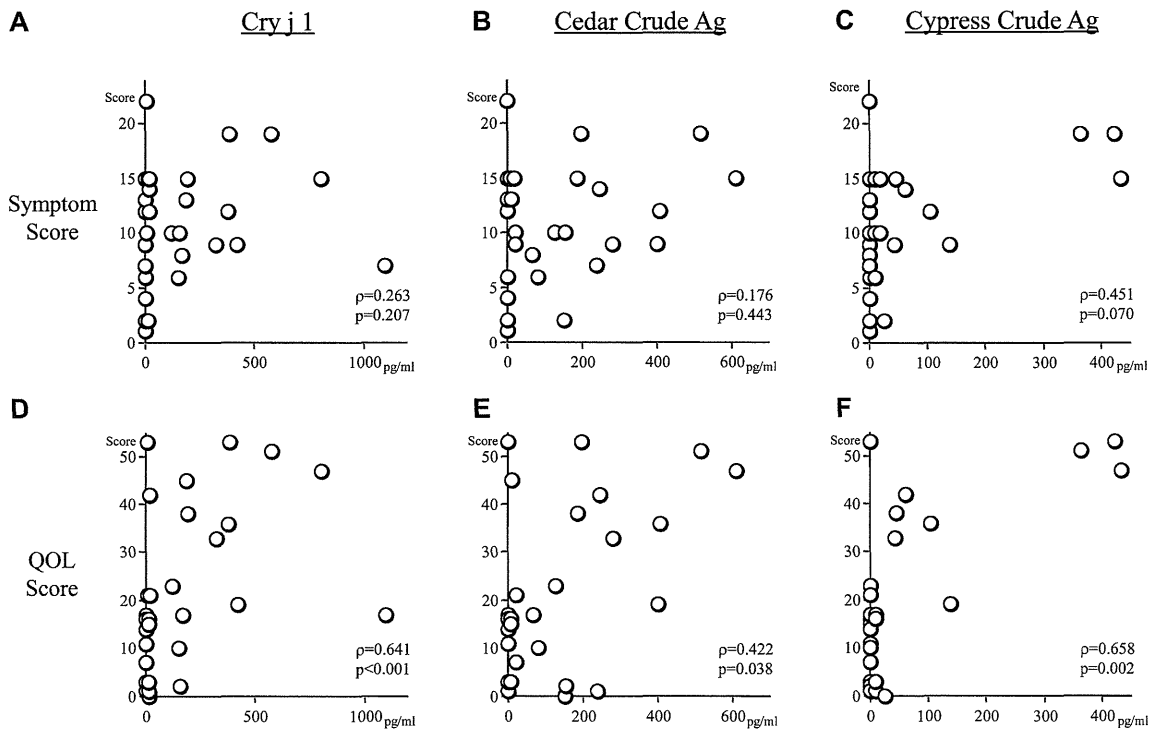


FIG E5. Relationship between naso-ocular symptoms **(A-C)** and QOL **(D-F)** during the peak season of cypress pollen dispersion and the amounts of IL-31 produced by PBMCs in response to Cry j 1 (Fig E5, *A* and *D*), cedar crude antigen (Fig E5, *B* and *E*), and cypress crude antigen (Fig E5, *C* and *F*). The ρ and *P* values were determined by using the Spearman correlation coefficient by rank. *Ag*, Antigen.

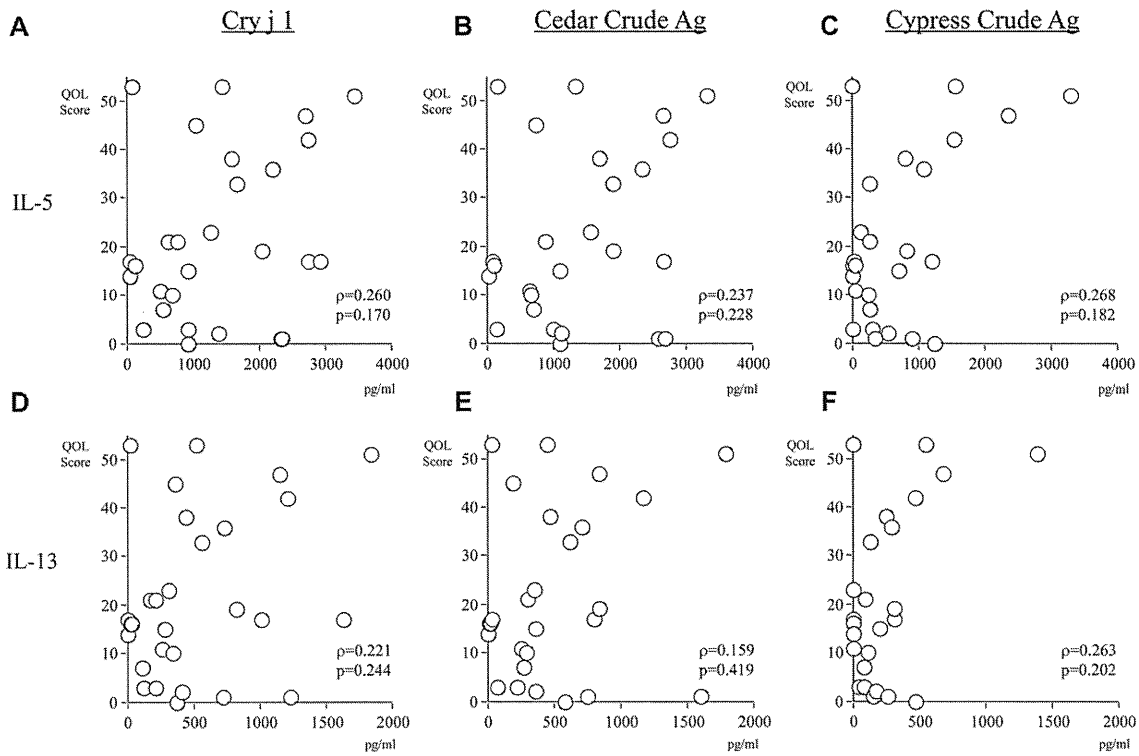


FIG E6. Relationship between QOL during the peak season of cypress pollen dispersion and the amounts of IL-5 **(A-C)** and IL-13 **(D-F)** produced by PBMCs in response to Cry j 1 (Fig E6, *A* and *D*), cedar crude antigen (Fig E6, *B* and *E*), and cypress crude antigen (Fig E6, *C* and *F*). The ρ and *P* values were determined by using the Spearman correlation coefficient by rank.

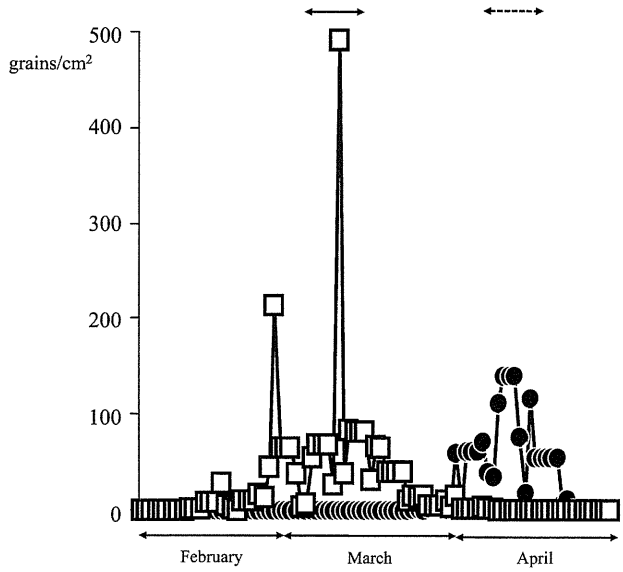


FIG E7. Fluctuation of cedar and cypress pollen dispersion from February to April 2009. *Open squares* and *filled circles* represent the counts of cedar and cypress pollen, respectively. Naso-ocular symptoms and rhinitis-related QOL were monitored during the peak season of cedar pollen dispersion (March 4-18, *solid line*) and cypress pollen dispersion (April 8-22, *dotted line*) by using Japanese rhinoconjunctivitis QOL questionnaire number 1.

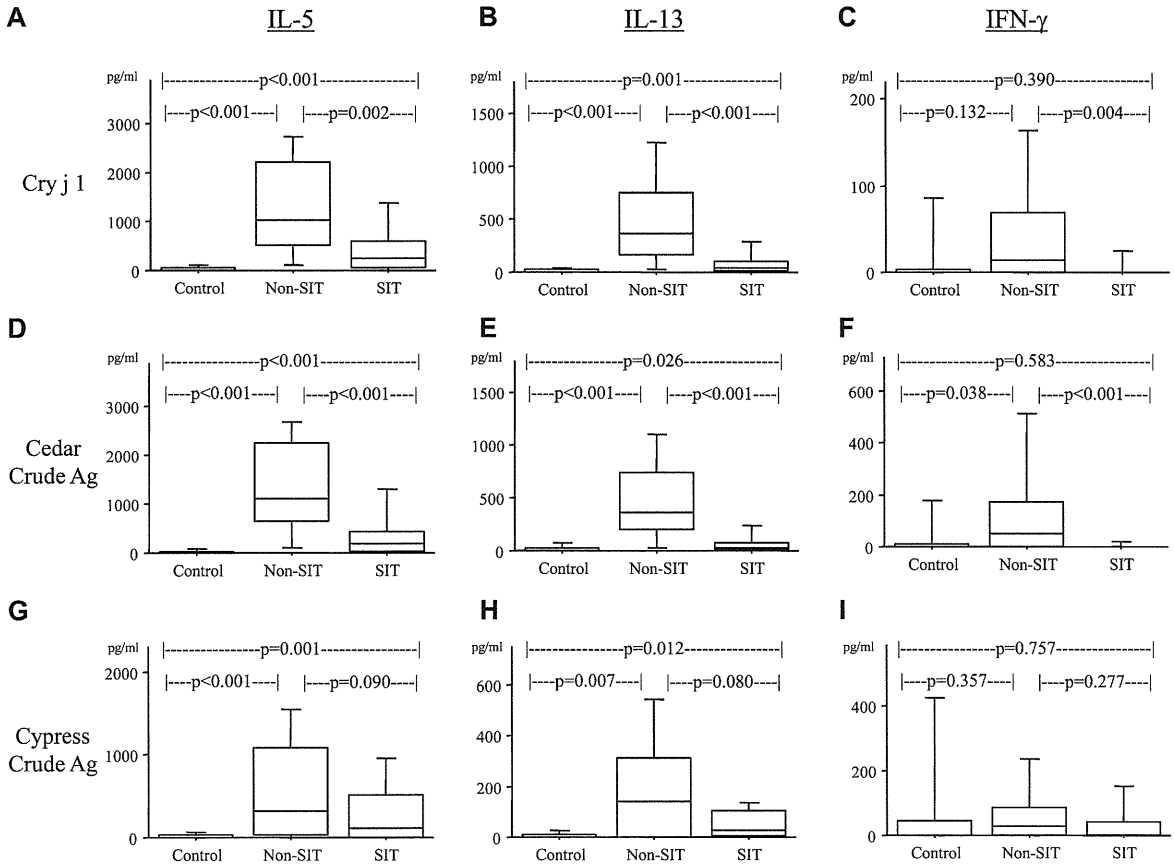


FIG E8. Production of T_H1 and T_H2 cytokines of PBMCs against stimulation with pollen antigens. PBMCs from healthy control subjects *(Control)*, patients with JCCP without specific immunotherapy with standardized extract of Japanese cedar pollen *(Non-SIT)*, and patients with JCCP with specific immunotherapy *(SIT)* were stimulated with Cry j 1 **(A-C)**, cedar crude antigen **(D-F)**, and cypress crude antigen **(G-I)** for 72 hours, and then the concentration of IL-5 (Fig E8, *A*, *D*, and *G*), IL-13 (Fig E8, *B*, *E*, and *H*), and IFN-γ (Fig E8, *C*, *F*, and *I*) in the supernatants was measured by means of ELISA. The *rectangle* includes the range from the 25th and 75th percentiles, the *horizontal line* indicates the median, and the *vertical line* indicates the range from the 10th to the 90th percentiles. *P* values were determined by using the Mann-Whitney *U* test. *Ag*, Antigen.

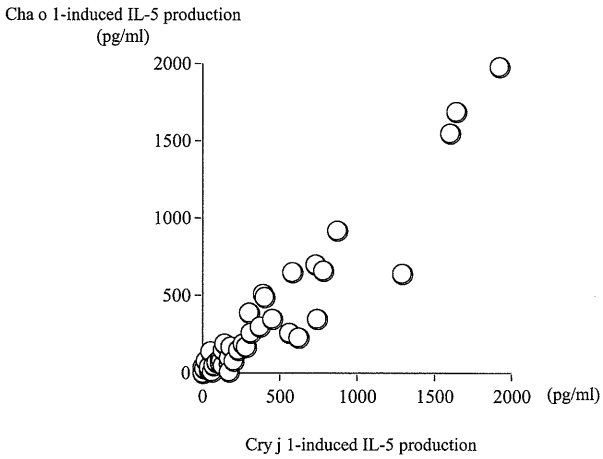


FIG E9. Relationship between Cry j 1– and Cha o 1–induced IL-5 production by PBMCs from patients with JCCP (n = 46).

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

Yasuhiro Uekusa, M.D., D.D.S., Ph.D.,[1,2] Ayako Inamine, Ph.D.,[1] Syuji Yonekura, M.D., Ph.D.,[1] Shigetoshi Horiguchi, M.D., Ph.D.,[1] Takashi Fujimura, Ph.D.,[1,3] Daijyu Sakurai, M.D., Ph.D.,[1] Heizaburo Yamamoto, M.D., Ph.D.,[1] Homare Suzuki, M.D., Ph.D.,[1] Toyoyuki Hanazawa, M.D., Ph.D.,[1] and 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.*

(Am J Rhinol Allergy 26, 92–96, 2012; doi: 10.2500/ajra.2012.26.3706)

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 AR[23–25] 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.

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

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

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, Uppsala, Sweden).

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 monoclonal antibodies were diluted to a concentration of 15 μg/mL in sterile, filtered (0.45 μm) PBS (pH 7.2), and added to nitrocellulose plates (Millititer; Millipore Corp., Bedford, MA) at 100

μL/well. The plates were incubated overnight at 4°C and unbound antibodies were 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 μL) 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 μL of biotinylated monoclonal antibodies (1 μg/mL) and incubation for 2 hours at room temperature. The plates were then washed and incubated for 90 minutes at room temperature with 100 μL 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 μL of 5-bromo-4-chloro-3-indoxyl phosphate and nitro blue tetrazolium chloride 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 μM of ionomycin, and 2 μM 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^+ cells and that of IL-10^+ cells among the CD4^+/CD25^+ 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 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/cm^2. 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).

Table 1 **Baseline characteristics of the subjects**

	AR$^+$	AR$^-$
Subjects	8	25
Sex (M/F)	4/4	16/9
Age, yr		
Mean	25.1 ± 7.7	26.3 ± 5.7
Range	18–41	19–37
sIgE titer (UA/mL)		
Mean	30.9 ± 28.8	10.7 ± 14.1
Range	2.62–79.5	0.77–57.7
tIgE titer (IU/mL)		
Mean	350.3 ± 284.0	390.48 ± 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.

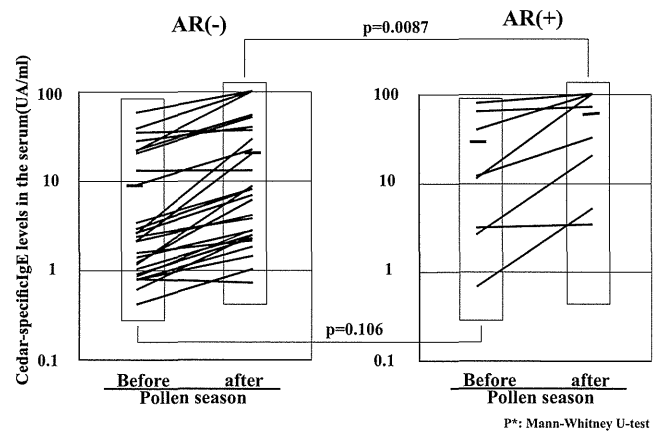


Figure 1. Cedar pollen–specific IgE levels in serum before and after pollen dispersal in 2008. The specific IgE (sIgE) titers before the pollen season did not differ significantly between subjects who did and not develop pollinosis. During the pollen season, the levels increased in almost all subjects.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

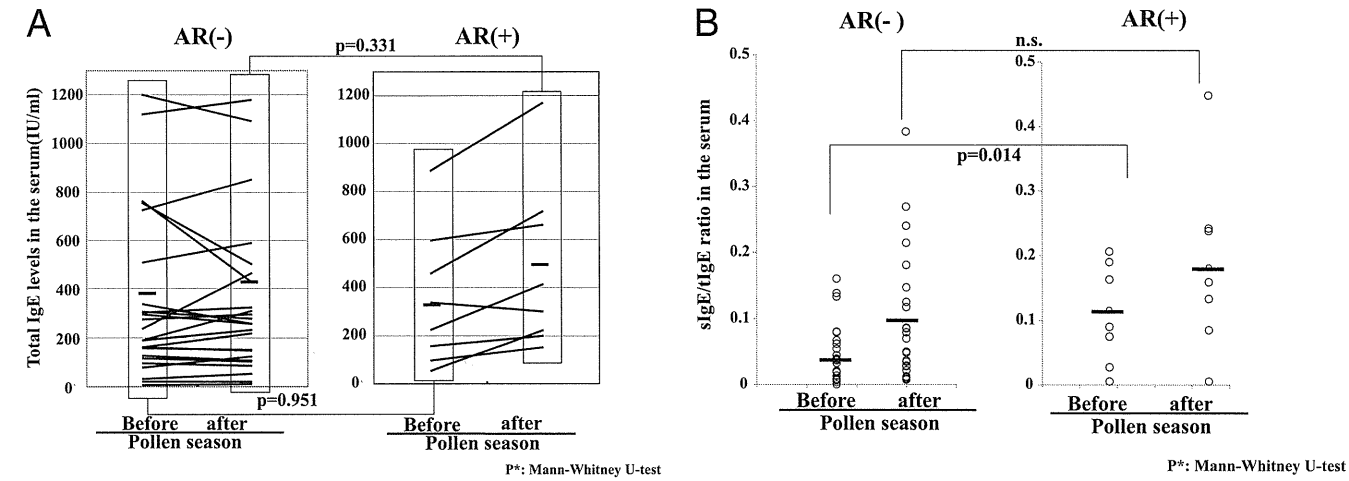


Figure 2. *(A) Total IgE (tIgE) levels in serum before and after pollen dispersal in 2008. The tIgE titers did not differ significantly between subjects who did and not develop pollinosis. During the pollen season, titers increased in almost all subjects and were not significantly different in the two groups. (B) The sIgE/tIgE ratios before and after pollen dispersal in 2008. The average ratio of Japanese cedar pollen sIgE/tIgE 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).*

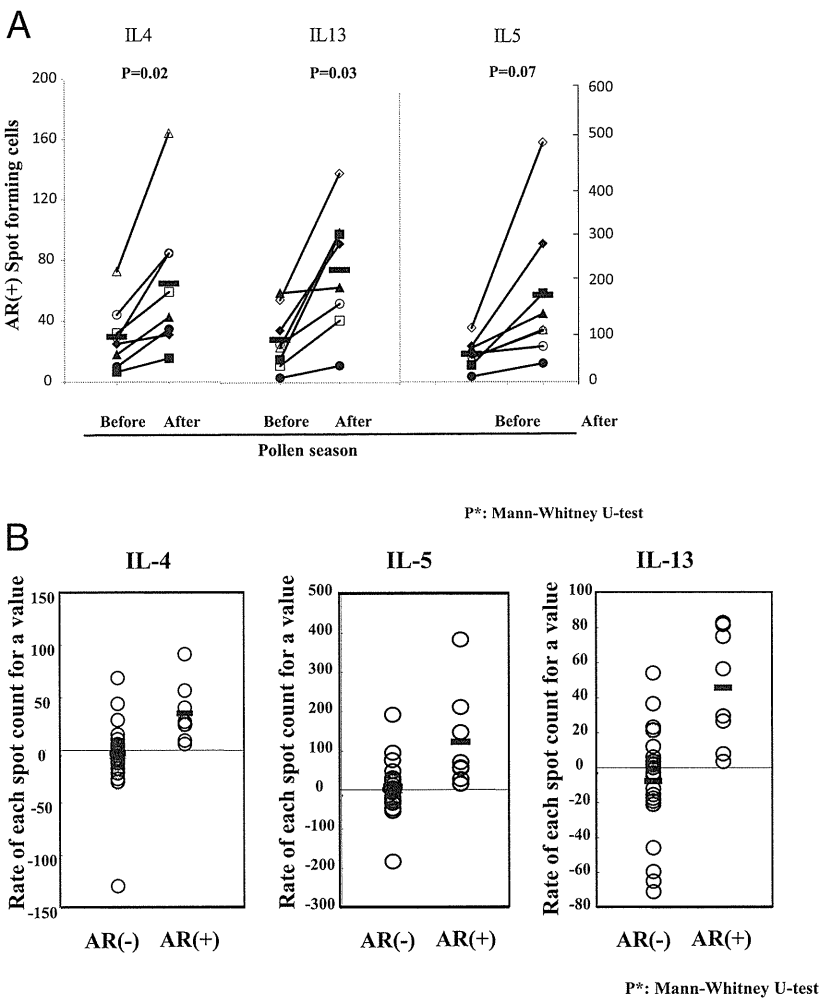


Figure 3. *Cry j 1 peptide–specific IL-4–, IL-5–, and IL-13–producing cells were counted by enzyme-linked immunosorbent spot (ELISPOT) assay before and after the pollen season in 2008. Samples at each time point were analyzed simultaneously. (A) IL-4, IL-5, and IL-13 spots after the pollen season in 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).*

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.

tIgE Levels in Serum

Total serum IgE levels are shown in Fig. 2 *A*. The tIgE 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.

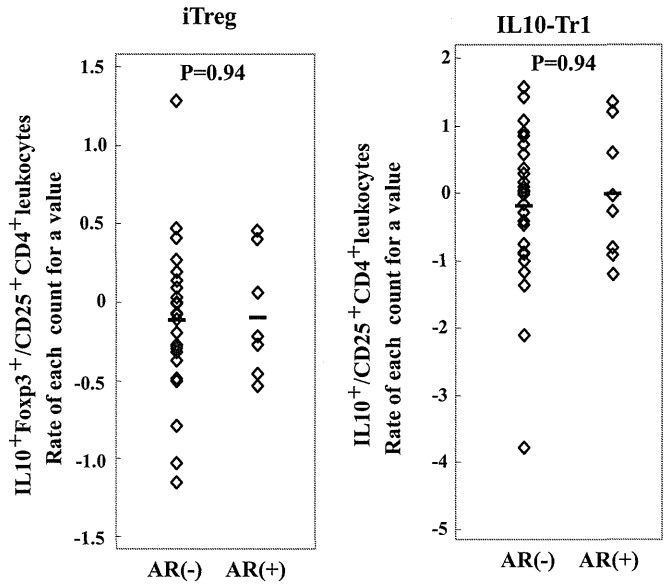


Figure 4. Detection of IL-10$^+$ Foxp3$^+$/CD25$^+$CD4$^+$ T cells and IL-10$^+$/CD25$^+$CD4$^+$ T cells in flow cytometric analysis of peripheral blood mononuclear cells (PBMCs). The relative changes from before to after the pollen season are shown for subjects who did and did not develop cedar pollinosis.

sIgE/tIgE Ratio

The average ratio of Japanese cedar pollen sIgE to tIgE in serum (sIgE/tIgE) 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*).

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/cm^2). 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/10^5 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 pol-

linosis, 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.

REFERENCES

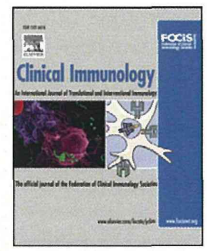
1. Bousquet J, Khaltaev N, and Cruz AA; Aria Workshop Group. World Health Organization: Allergic rhinitis and its impact on asthma (ARIA) 2008 update. Allergy 63(suppl 86):8–160, 2008.
2. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. Lancet 351:1225–1232, 1998.
3. Michael S, and Blaiss MD, Allergic rhinitis: Direct and indirect costs. Allergy Asthma Proc 31:375–380, 2010.
4. Eli OM, Robert N, Jennifer D, et al. Sleep, quality of life, and productivity impact of nasal symptoms in the United States: Findings from the Burden of Rhinitis in America survey. Allergy Asthma Proc 30:244–254, 2009.
5. So YK, Seok-Jun Y, Min-Woo J, et al. Economic burden of allergic rhinitis in Korea. Am J Rhinol Allergy 24:e110–e113, 2010.
6. Kurz T, Altmueller J, Strauch K, et al. A genome-wide screen on the genetics of atopy in a multiethnic European population reveals a major atopy locus on chromosome 3q21.3. Allergy 60:192–199, 2005.
7. Barnes KC, and Marsh DG. The genetics and complexity of allergy and asthma. Immunol Today 19:325–332, 1998.
8. Kompauer I, Henrich J, Wolfman G, and Linseisen J. Association of carotenoids, tocopherols and vitamin C in plasma with allergic sensitization in adults. Public Health Nutr 9:472–479, 2006.
9. Am JS, Swartz J, Lilja G, et al. Atopy in children of families with an anthroposophic lifestyle. Lancet 353:1485–1488, 1999.
10. Lee YL, Shaw CK, Su HJ, et al. Climate, traffic-related air pollutions and allergic rhinitis prevalence in middle-school children in Taiwan. Eur Respir J 21:964–970, 2003.
11. Strachan DP. Hay fever, hygiene, and household size. BMJ 299:1259–1260, 1989.
12. Bodtger U, Poulsen LK, and Linneberg A. Rhinitis symptoms and IgE sensitization as risk factors for development of later allergic rhinitis in adults. Allergy 61:712–716, 2006.
13. Pastorello EA, Incorvaia C, Ortolani C, et al. Studies on the relationship between the level of specific IgE antibodies and the clinical expression of allergy: I. Definition of levels distinguishing patients with symptomatic from patients with asymptomatic allergy to common aeroallergens. J Allergy Clin Immunol 96:580–587, 1995.
14. Niemeijer NR, Fluks AF, and de Monchy JG. Optimization of skin testing. II. Evaluation of concentration and cutoff values, as compared with RAST and clinical history, in a multicenter study. Allergy 48:498–503, 1993.
15. Mabry RL. Rhinitis of pregnancy. South Med J 79:965–971, 1986.
16. Okamoto Y, Horiguchi S, Yamamoto H, et al. Present situation of cedar pollinosis in Japan and its immune responses. Allergol Int 58:155–162, 2009
17. Okawa T, Konno A, Ymakoshi T, et al. Analysis of natural history of Japanese cedar pollinosis. Int Arch Allergy Immunol 131:39–45, 2003.
18. Schoefer Y, Scäfer T, Meisinger C, et al. Predictivity of allergic sensitization (RAST) for the onset of allergic disease in adults. Allergy 63:81–86, 2008.
19. Fagan JK, Scheff PA, Hryhorczuk D, et al. Prevalence of asthma and other allergic disease in an adolescent population: Association with gender and race. Ann Allergy Asthma Immunol 86:177–184, 2001.
20. Lee JH, Haselkorn T, Chipps BE, et al. Gender difference in IgE-mediated allergic asthma in the epidemiology and natural history of asthma: Outcomes and Treatment Regimens (TENOR) study. J Asthma 43:179–184, 2006.
21. Bertelsen RJ, Instanes C, Granum B, et al. Gender differences in indoor allergen exposure and association with current rhinitis. Clin Exp Allergy 40:1388–1397, 2010.
22. Han D, Wang C, Lou W, et al. Allergen-specific IL-10-secreting type 1 T regulatory cells, But not $CD4^+CD25^+Foxp3^+$ T cells, are decreased in peripheral blood of patients with persistent allergic rhinitis. Clin Immunol 136:292–301, 2010.
23. Wahn U, Lau S, Bergmann R, et al. Indoor allergen exposure is a risk factor for sensitization during the first three years of life. J Allergy Clin Immunol 99:763–769, 1997.
24. Brozek JL, Bousquet J, Baena-Cagnani CE, et al. Allergic Rhinitis and Its Impact on Asthma (ARIA) guidelines: 2010 Revision. J Allergy Clin Immunol 126:466–476, 2010.
25. Holt PG, and Sly PD. Prevention of allergy respiratory disease in infants: Current aspects and future perspectives. Curr Opin Allergy Clin Immunol 7:547–555, 2007.
26. Horiguchi S, Tanaka Y, Uchida T, et al. Seasonal changes in antigen-specific Th clone sizes in patients with Japanese cedar pollinosis: A 2-year study. Clin Exp Allergy 38:405–412, 2008.
27. Fujimura T, Yonekura S, Taniguchi Y, et al. The induced regulatory T-cell level, defined as the proportion of $IL10^+Foxp3^+$ cells among $CD25^+CD4^+$ leukocytes, is a potential therapeutic biomarker for sublingual immunotherapy: A preliminary report. Int Arch Allergy Immunol 153:378–387, 2010.
28. Di Lorenzo G, Mansueto P, and Pacor ML, Evaluation of serum s-IgE/total IgE ratio in predicting clinical response to allergen-specific immunotherapy. J Allergy Clin Immunol 123:1103–1110, 2009.
29. Fujimura T, and Okamoto Y. Antigen-specific Immunotherapy against allergic rhinitis: The state of art. Allergol Inter 59:21–31, 2010.
30. Palomares O, Yaman G, Azkur AK, et al. Role of Treg in immune regulation of allergic disease. Eur J Immunol 40:1232–1240, 2010.
31. Emel K, Ayse A, Zafer G, et al. The effects of natural pollen exposure on inflammatory cytokines and their relationship with nonspecific bronchial hyperresponsiveness in seasonal allergic rhinitis. Allergy Asthma Proc 31:126–131, 2010. □

Clinical Immunology (2012) 143, 170–179

available at www.sciencedirect.com

Clinical Immunology

www.elsevier.com/locate/yclim



Sublingual administration of *Lactobacillus paracasei* KW3110 inhibits Th2-dependent allergic responses via upregulation of PD-L2 on dendritic cells

Ayako Inamine [a], Daijyu Sakurai [a], Shigetoshi Horiguchi [a], Syuji Yonekura [a], Toyoyuki Hanazawa [a], Hiroyuki Hosokawa [b], Asaka Matuura-Suzuki [a], Toshinori Nakayama [b], Yoshitaka Okamoto [a,*]

[a] *Department of Otolaryngology, Head and Neck Surgery (J2), Graduate School of Medicine, Chiba University, Chiba, Japan*
[b] *Department of Immunology (H3), Graduate School of Medicine, Chiba University, Chiba, Japan*

Received 6 December 2011; accepted with revision 16 January 2012
Available online 27 January 2012

KEYWORDS
Allergy;
Dendritic cells;
Vaccination;
Sublingual administration

Abstract Lactic acid bacteria have potential in immunomodulation therapy, but their clinical efficacy and underlying mechanisms are unclear. We aimed to clarify the anti-allergic immune responses induced by intragastric and sublingual administration of heat-killed *Lactobacillus paracasei* KW3110 and *Lactobacillus acidophilus* L-92. The KW3110 strain (but not the L-92 strain) enhanced ovalbumin (OVA)-induced expression of CCR-7 and PD-L2 in murine dendritic cells (DCs), and strongly inhibited IL-5 and IL-13 production *in vitro* in co-cultures with Th2-skewed $CD4^+$ T cells from DO11.10 transgenic mice. Sublingual administration of low-dose KW3110 (but not L-92) to OVA-sensitized mice selectively suppressed serum IgE production and Th2 cytokine expression in cervical lymph nodes, and significantly improved symptoms after OVA provocation *in vivo*. KW3110 probably accelerates DC migration into the regional lymph nodes and inhibits Th2 cytokine production through enhanced CCR-7 and PD-L2 expression. Thus, sublingual KW3110 administration may be effective in reducing allergic inflammation.

Abbreviations: PBMC, Peripheral blood mononuclear cell; SLIT, Sublingual immunotherapy; DC, Dendritic cell; OVA, Ovalbumin; BM, Bone marrow; LPS, Lipopolysaccharide; CLN, Cervical lymph node; ILN, Inguinal lymph node; ELISA, Enzyme-linked immunosorbent assay; ELISPOT, Enzyme-linked immunospot; TLR, Toll-like receptor; LGG, *Lactobacillus rhamnosus* GG; FACS, Fluorescein-activated cell sorter; FITC, Fluorescein isothiocyanate; GM-CSF, Granulocyte macrophage colony-stimulating factor; TCR, T cell receptor; OD, Optical density

* Corresponding author at: Department of Otolaryngology, Head and Neck Surgery, Graduate School of Medicine Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. Fax: +81 43 226 3442.
E-mail addresses: ainamine@faculty.chiba-u.jp (A. Inamine), sakuraidai@faculty.chiba-u.jp (D. Sakurai), horiguti@faculty.chiba-u.jp (S. Horiguchi), syonekura@faculty.chiba-u.jp (S. Yonekura), thanazawa@faculty.chiba-u.jp (T. Hanazawa), hosohiro@chiba-u.jp (H. Hosokawa), asakamatsu@office.chiba-u.jp (A. Matuura-Suzuki), tnakayama@faculty.chiba-u.jp (T. Nakayama), yokamoto@faculty.chiba-u.jp (Y. Okamoto).

1521-6616/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.clim.2012.01.008

1. Introduction

An increased prevalence of allergic rhinitis has been observed in many countries over the recent years [1]. A variety of medications have been used to relieve the symptoms of this condition; however, these drugs do not treat the underlying disease and have a high risk of adverse events, particularly when taken over a long period [2]. Antigen-specific immunotherapy can effectively change the natural course of allergic disease, prevent the development of other allergic diseases, and reduce new allergic sensitization [3]. However, conventional subcutaneous administration necessitates frequent visits to the physician and is associated with a risk, albeit low, of anaphylactic shock [4].

The efficacy of lactic acid bacteria in suppressing the development of allergic diseases has been demonstrated [5]. For example, when expectant mothers were treated with *Lactobacillus rhamnosus* GG (LGG), and their infants were also treated with LGG from birth to 6 months of age, there was inhibition of the development of atopic dermatitis at ages 2 and 4 years [6]. In a study of infants with milk allergy treated with LGG, placebo or other *Lactobacillus* strains, LGG significantly enhanced IFN-γ production from peripheral blood mononuclear cells (PBMCs) [6]. However, in another study, there was no evidence of significant clinical benefit or differences in cytokine production from PBMCs in infants treated with LGG compared to a placebo [7].

Xiao et al. [8] observed marked improvements in nasal symptoms and modulation of Th2-skewed immune responses after 13 weeks of administration of the probiotic strain *Bifidobacterium longum* for treatment of Japanese cedar pollinosis. There is no other evidence of benefit after 22 weeks of administration of LGG for treatment of birch pollinosis [7]. The use of different probiotics and the study methodologies, such as dose, period of administration, and study sample size may explain these contradictory results. Intestinal health and factors such as diet and antibiotic therapy can also have a significant influence, which makes it difficult to evaluate the roles of probiotics [8,9].

In this study, we examined 2 strains of heat-killed lactic acid bacteria, *Lactobacillus paracasei* KW3110 and L-92, which are widely used in Japan and are reported to produce unique and different immune responses *in vitro*. KW3110 induces higher levels of IL-12 *in vitro* than the LGG strain, making it a more effective strain [9]. KW3110 may inhibit Th2 cytokine-mediated allergic inflammation through mechanisms independent of toll-like receptors (TLRs) [10,11], since the induction of IL-12 is not abrogated in bone marrow (BM)-derived dendritic cells (DCs) in TLR2-, TLR4- and TLR9-deficient mice [12]. L-92 activates DCs in a TLR2-dependent manner [13] and may exhibit anti-allergic activity through induction of regulatory T cells *in vivo* [14]. We administered the *Lactobacillus* strains directly to the oral mucosa, rather than as probiotics, and examined the influence of the KW3110 and L-92 strains on allergic responses in antigen-sensitized mice *in vivo* and *in vitro*.

We have previously observed higher CCR-7 and PD-L2 expression in mature DCs upon stimulation with KW3110 but not with L-92, which accelerated DC migration to draining lymph nodes and increased inhibitory signals for Th2 cytokine production. Similar immunomodulation was observed in cervical lymph nodes (CLNs), which play an important role in allergic rhinitis as draining lymph nodes, after sublingual administration of KW3110 *in vivo*. This therapeutic approach may therefore be effective for allergic rhinitis.

2. Material and methods

2.1. Mice

Seven-week-old female BALB *c* mice were purchased from SLC Inc. (Hamamatsu, Japan) and were maintained under specific pathogen-free conditions. The use of these mice was approved by the Chiba University Institutional Animal Care and Use Committee and the experiments were conducted in conformity with the guidelines of the committee.

2.2. Reagents and medium

The heat-killed KW3110 [11] and L-92 [14] strains were obtained from the Central Laboratories for Frontier Technology, Kirin Holdings Co. (Kanagawa, Japan), and from the R&D Center, Calpis Co. (Yokohama, Japan) respectively. Ovalbumin (OVA) (grade 5) was purchased from Sigma-Aldrich (St. Louis, MO, USA). We used RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FCS, L-glutamine (2 μM), penicillin (100 $U.mL^{-1}$), streptomycin (100 $\mu g.mL^{-1}$), HEPES (10 mM), 2-mercaptoethanol (55 μM), 1% non-essential amino acids, and 1 mM sodium pyruvate (all from Gibco BRL, Grand Island, NY, USA) in the cell culture experiments. OVA was dissolved in endotoxin-free D-PBS (Wako Pure Chemical Industries, Osaka, Japan), which had an endotoxin level below the detection limit (0.05 ELISA units per milligram protein). The reagents used for stimulation were also tested for endotoxin contamination; these also had levels below the detection limit.

2.3. Maturation of DCs

DCs from murine BM were cultured in a 100-mm bacteriological petri dish with 10 $ng.mL^{-1}$ granulocyte macrophage colony-stimulating factor (GM-CSF) (PeproTech, Rocky Hill, NJ) [15]. The medium was replaced twice. The immature DCs were stimulated with 1 $mg.mL^{-1}$ of OVA in endotoxin-free PBS with or without 1 $\mu g.mL^{-1}$ KW3110 or L-92, and 10 $\mu g.mL^{-1}$ LPS (Lipopolysaccharide) (O111:B4, Sigma-Aldrich), and cultured for 24 h in 24-well plates at 1×10^6 cells per well. The DCs were analyzed by fluorescein-activated cell sorter (FACS) analysis of surface markers (FACSCalibur; Becton Dickinson, Sunnyvale, CA).

2.4. Phagocytosis by DCs *in vitro*

KW3110 or L-92 (1 mg of each) was suspended in 1 mL of 100 mM carbonate buffer (pH 9.5), reacted with fluorescein 5(6)-isothiocyanate (100 $\mu g.mL^{-1}$) (Sigma-Aldrich) at 37 °C for 60 min, and then washed with sterile PBS [16]. Fluorescein isothiocyanate (FITC)-labeled *Lactobacillus* (1 $\mu g.mL^{-1}$) was cultured with immature DCs (1×10^6 $cells.mL^{-1}$) for 24 h. After washing with PBS, the DCs were fixed with a cold methanol:acetone (1:1) solution for 10 min and then stained with biotin-labeled CD11c (N418; BioLegends, San Diego, CA, USA), followed by Cy5-coupled streptavidin. For