5]. Cedar forests cover nearly 18% of the total land area of Japan, while Japanese cypress is concentrated in the Kanto region and the western part of the country. Both cedar and Japanese cypress produce enormous amounts of pollen which is dispersed over many kilometers and reaches major cities, including Tokyo and Osaka, causing widespread pollinosis. Cedar pollen dispersal precedes Japanese cypress pollen dispersal, and approximately 70% of patients with cedar pollinosis are also allergic to Japanese cypress pollen. Cedar pollen dispersal starts in early February and reaches a peak between late February and early March, and this is followed by the dispersal of Japanese cypress pollen, which reaches a peak from late March to early April, with some variation due to changes in the climate each year [6]. The pollen dispersal season lasts for more than 10 weeks in and around the area of Chiba.

Outside the pollen dispersal season, patients with cedar and Japanese cypress pollinosis who do not have rhinitis caused by other allergens in general exhibit normal nasal mucosa with few or no symptoms. Repeated exposure to pollen induces allergic inflammation and increases hypersensitivity of the nasal mucosa, and early intervention against mild pollinosis just after the start of the pollen dispersal season may have a significant effect on the severity of symptoms when pollen dispersal is at its peak. In Japan, the Ministry of the Environment makes a detailed prediction of the date around which cedar pollen dispersal is likely to start, making it easy to assess the effects of early intervention in patients with pollinosis due to cedar and Japanese cypress.

Leukotriene receptor antagonists (LTRA) have been shown to be effective in controlling nasal inflammation [7, 8] by their ability to inhibit eosinophil secretion in the airway [9, 10]. Recent studies have shown that LTRA are as effective as antihistamines but less effective than nasal steroids [11–13]. Nasal steroids reduce sneezing, nasal secretion as well as nasal obstruction. However, the compliance for use of nasal steroids is sometimes poor [14] and their market share is much smaller than that for other oral anti-allergic medications in Japan, because patients seem to prefer such non-sensory attribute, painless route of administration [15, 16].

LTRA do not exert any central nervous system depression or adrenal suppressive effects and may be more suitable for early interventional use especially in patients with milder symptoms. In order to determine the effects of early intervention with LTRA on cedar and Japanese cypress pollinosis, we conducted a double-blind, placebo-controlled trial in subjects allergic to both pollens. Either LTRA or placebo was administered to subjects immediately before the start of the pollen dispersal season and continued throughout the pollen season. All subjects received nasal steroids after the initial treatment with LTRA. Symptom and quality of life (QOL) scores were monitored during the pollen and before the dispersal season and after concomitant therapy with nasal steroids and LTRA.

Subjects and Methods

Subjects

The study population comprised 60 subjects (30 males and 30 females), ranging in age from 20 to 65 years, who were otherwise healthy, but who had a clinical history of moderate/severe Japanese cedar and cypress pollinosis for at least 3 consecutive cedar and cypress pollen seasons. The subjects lived in and around Chiba City where the pollen spread would be expected to be consistent. The diagnosis of cedar and cypress pollinosis was based on clinical history, positive allergen-specific skin tests (wheal diameter $\geq$10 mm) to a standardized cedar pollen extract (Torii Pharmaceutical Co., Tokyo, Japan), and a serum cedar and cypress pollen-specific IgE level score $\geq$2 by a CAP radioallergosorbent test (SRL Inc., Tokyo, Japan). Exclusion criteria were complication of moderate/severe perennial allergic rhinitis with a need for treatment, a history of severe asthma, use of anti-allergic drugs within 4 weeks, and a prior history of any allergen-specific immunotherapy, including for cedar pollen. Pregnant women or those at risk of pregnancy were also excluded. The study was conducted at Chiba University Hospital in compliance with the Ethical Guidelines for Clinical Studies and Good Clinical Practice and the Declaration of Helsinki (2000 revision). The Ethics Committee of Chiba University approved the protocol, and written informed consent was obtained from each subject prior to his or her participation in the study.

Methods

Capsules containing 112.5 mg of pranlukast hydrate or placebo were used in the study. The study schedule is shown in figure 1. Prior to the study, patients were interviewed regarding their medical history and underwent the skin test for cedar pollen extract and a CAP radioallergosorbent test in late January 2007 to measure specific serum antibodies against cedar and Japanese cypress pollen. Administration of LTRA or placebo was initiated before the start of the cedar pollen dispersal season, which had been forecast to be in early February. Two capsules were administered orally twice a day after breakfast and dinner for 4 weeks (hereafter referred to as 'pretreatment' period). During the latter 2 weeks of this period, subjects were allowed to use an antihistamine (loratadine, 1 capsule per day), other nasal vasoconstriction drops (tetrahydrozoline hydrochloride, maximum 2 drops to each nasal cavity per day and less than 7 days successively), or disodium cromoglycate eye drops (maximum 4 drops to each eye) at their own discretion, based on the severity of symptoms. Subsequently, all subjects took nasal steroids (fluticasone propionate) and LTRA for 4 weeks (main dispersal treatment period) in accordance with ARIA [2] and the Practical Guidelines for the Management of Allergic Rhinitis in Japan [4], again based on the severity of symptoms.

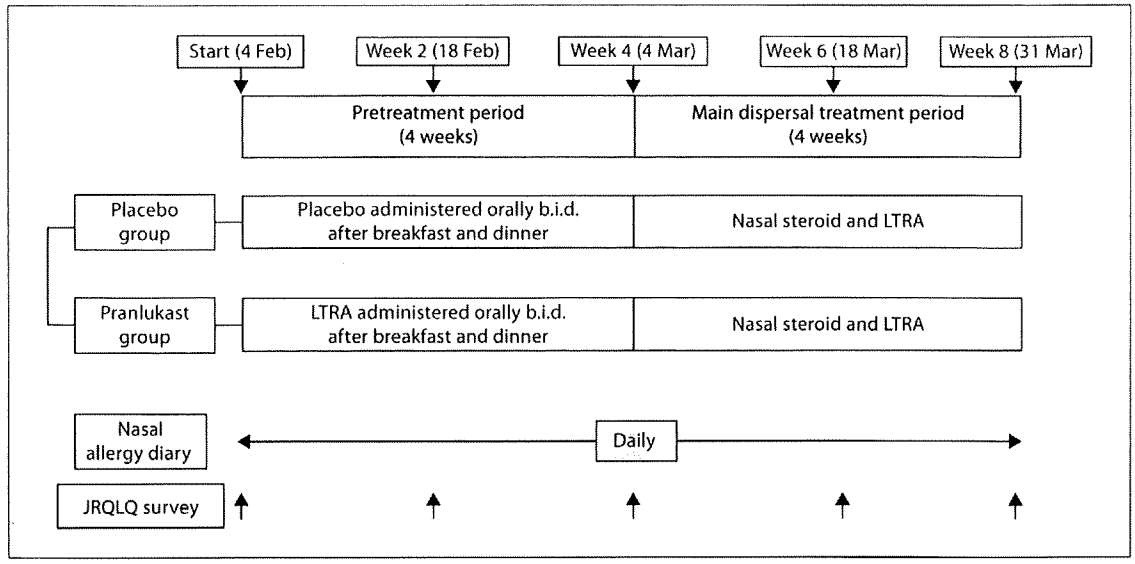

Fig. 1. Study schedule. b.i.d. = Twice daily.

Table 1. Severity of nasal symptoms

Parameter	Severity				
	++++	+++	++	+	–
Paroxysmal sneezing, times/day	≥21	11–21	6–10	1–5	0
Runny nose (nose blowing frequency) times/day	≥21	11–21	6–10	1–5	0
Nasal congestion	complete congestion, all day	very severe nasal congestion with frequent oral breathing	severe nasal congestion with occasional oral breathing	no oral breathing but nasal congestion	none

Adapted from the Practical Guideline for the Management of Allergic Rhinitis in Japan, 2005 [5].

Some subjects (n = 30) received LTRA throughout the study period (LTRA group). However, a group of other subjects (n = 30) received placebo during the pretreatment period and LTRA during the main treatment period (placebo group). The sample size was determined based on previous studies of LTRA on the change and variance of clinical symptoms [17]. A nasal allergy diary was written daily, and Japan Rhinoconjunctivitis Quality of Life Questionnaire (JRQLQ) survey sheets [18, 19] were completed every 2 weeks until completion of the study. For assignment of subjects to groups, limited randomization was performed in subgroups of 6 age- and sex-matched subjects each, of whom 3 were assigned to the LTRA group and 3 were assigned to the placebo group. A controller who was not directly involved in the study was responsible for group allocation. A group allocation number was given to each subject. This information was closely guarded by the controller and by 1 member of the ethical committee not directly involved in the study.

During the study period, all study subjects recorded their use (dose and frequency) of permitted concomitant medications (listed above) in a nasal allergy diary. Use of other drugs considered unlikely to affect the study was also allowed.

Cedar and Japanese cypress pollen dispersal was measured with a Durham sampler installed on the roof top of one of the buildings in the School of Medicine, Chiba University.

Nasal symptoms, eye symptoms, symptom scores, medication scores and symptom-medication scores were evaluated from the nasal allergy diary using the following criteria. For nasal symptoms, the severity of paroxysmal sneezing (number of sneezes per day), runny nose (number of times of blowing the nose per day), nasal congestion, and the degree of interference with daily life were evaluated on a 5-point scale (0–4) using a modified Okuda classification [4, 20] (table 1). Symptom scores for classification of the severity of nasal symptoms were calculated using the same classification. The daily total nasal symptom score was expressed

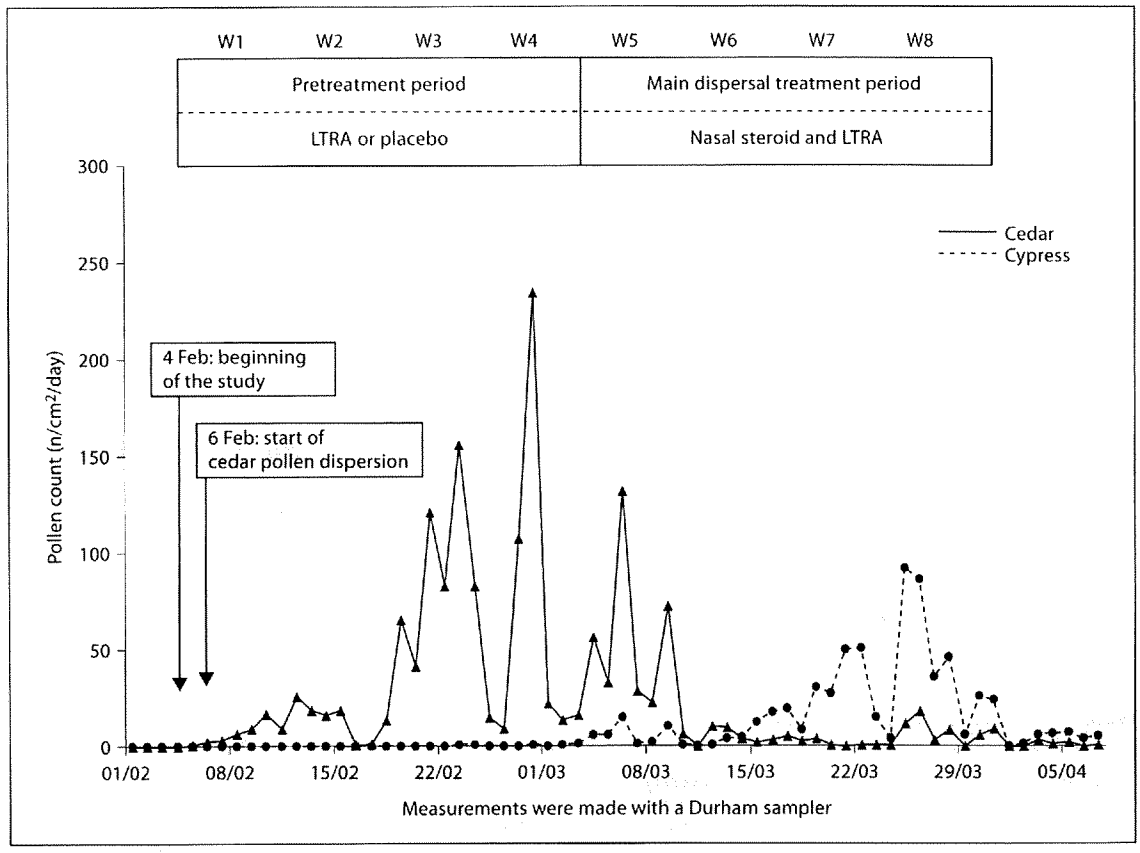

Fig. 2. Dispersal of cedar and Japanese cypress pollen in 2007 and study schedule.

as the highest sore of nasal symptoms. For eye symptoms, itching and watering were evaluated using a 4-point scale. The use of other medications was also scored and recorded according to the characteristics of the drug and the duration of usage, based on the following guidelines: anti-histamines, mast cell stabilizers and vasoconstrictors scored as 1, topical nasal steroids scored as 2, and the symptom-medication score was determined by adding the symptom score and the medication score. The score for each QOL item was also evaluated on a 5-point scale (0–4). In addition, the percent improvement in nasal symptoms was analyzed and expressed as the ratio of the patients who had improved nasal symptoms ≥ 1 in week 8 at the end of the study compared with week 4 before the concomitant therapy with nasal steroids. The symptom-medication score was used as the primary outcome parameter and other items were used as secondary parameters.

Statistical Analysis

After completion of the study (clinical and laboratory), a biostatistician who had not been involved in carrying out the clinical trial, analyzed the data. After completing the analysis, the allocation identification numbers for the active and placebo groups were accessed. Data comparisons were performed using 2-tailed tests at a significance level of 5%, using a χ^2 test, the Fisher exact test, the Mann-Whitney U test, a 2-sample t test, a paired t test, and the Wilcoxon test in SAS version 8.02 (SAS Inc., Cary, N.C., USA).

Results

Dispersal of Cedar and Japanese Cypress Pollen

Measurements with a Durham sampler (fig. 2) indicated that 6 February was the start of the cedar pollen dispersal season, based on a pollen count of $\geq 1/cm^2$/day. After 19 February, a pollen count $\geq 20/cm^2$/day was obtained on most days, which dropped to $<10/cm^2$/day after 10 March, marking the end of the dispersal season. Japanese cypress pollen was observed in the middle of March and reached a count of $>20/cm^2$/day on most days after 19 March until dispersal ended in early April.

Subjects

Four subjects withdrew from the study for personal reasons, and not because of any adverse effects. All other subjects exhibited full compliance with the study protocol. Thus, a total of 56 subjects were included for complete evaluation. The LTRA group comprised 29 subjects (mean age 36.1 years and cedar pollen RAST score 3.9). The placebo group comprised 27 subjects (mean age 33

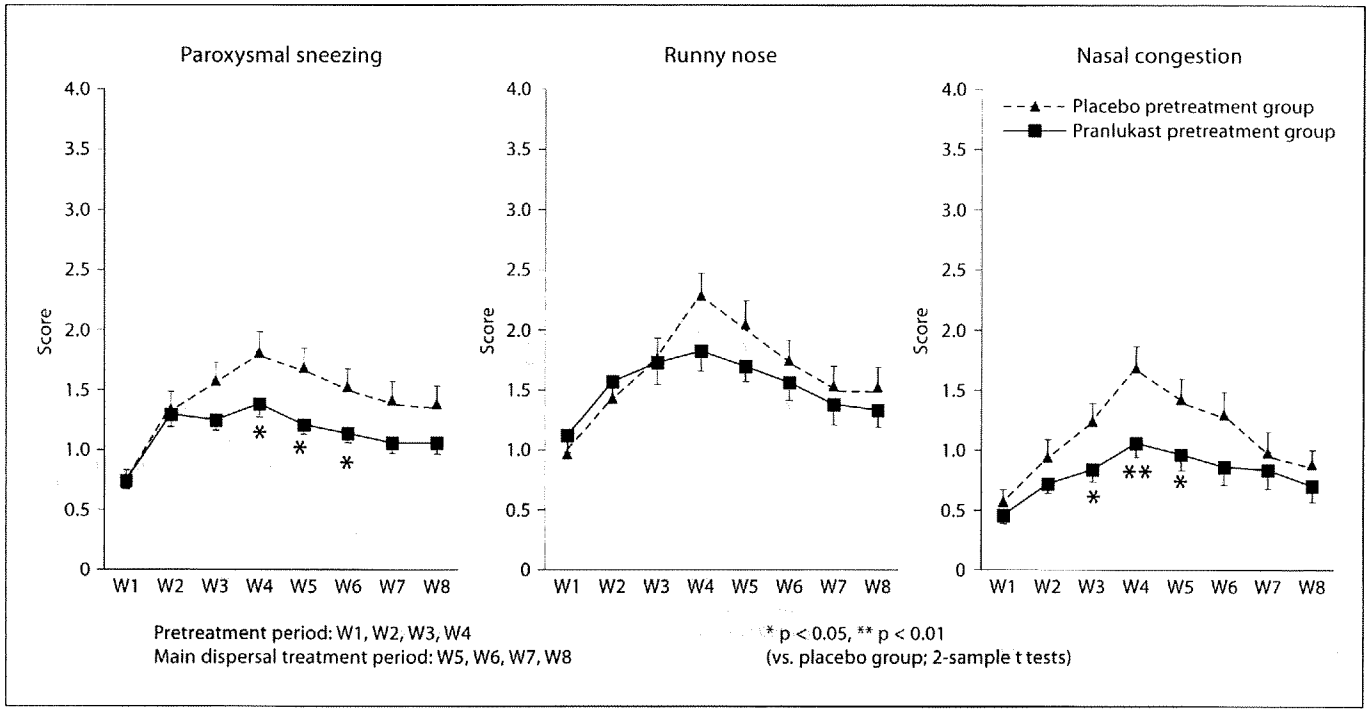

Fig. 3. Mean weekly score for each nasal symptom.

years and cedar pollen RAST score 3.5). There were no significant differences between the 2 study groups for age at disease onset, disease duration, or any subsequent complications.

Treatment Effects

The mean nasal symptom scores for each week of the pollen season are shown in figure 3. In both groups, symptoms worsened as cedar pollen dispersal increased and then improved after week 5, after the start of nasal steroid drops. In the LTRA group, nasal symptoms were mild from week 3 until the end of the study, and sneezing and nasal congestion scores were significantly lower in the LTRA group than in the placebo group between weeks 4 and 6 and weeks 3 and 5, respectively.

The total nasal symptom score increased in both groups from the start of the cedar pollen dispersal season and decreased in week 5, after the use of nasal steroids at the peak of the cedar dispersal season (fig. 4). Mean symptom scores were significantly lower in the LTRA group than in the placebo group in weeks 4 and 5 (fig. 4). Medication and symptom-medication scores also increased following the start of cedar pollen dispersal and decreased in week 5, after the start of nasal steroid therapy. These scores were lower in the LTRA group than in the placebo group during the pretreatment period, although the differences were not significant (data not shown). There were no significant differences in eye itching or watering scores between the groups (data not shown).

The degree of interference with daily life increased in both groups following the onset of cedar pollen dispersal and decreased in week 5 following the start of nasal steroid therapy. The score in the LTRA group was significantly lower than that in the placebo group in week 4 (fig. 4).

A comparative analysis of the improvement in nasal symptom score in week 8 (at the end of the study) and in week 4 (before the concomitant therapy with nasal steroids) is shown in table 2. The percent improvement in nasal congestion was significantly higher in LTRA-pretreated patients (69.0%) than in placebo-pretreated patients (40.7%).

JRQLQ Scores

For 17 QOL items, each mean QOL score generally increased by ≥0.5 points after pollen dispersal (data not shown) and improved for all items after the start of concomitant therapy at week 5 with nasal steroid drops (fig. 5). Although scores for all items in the LTRA group

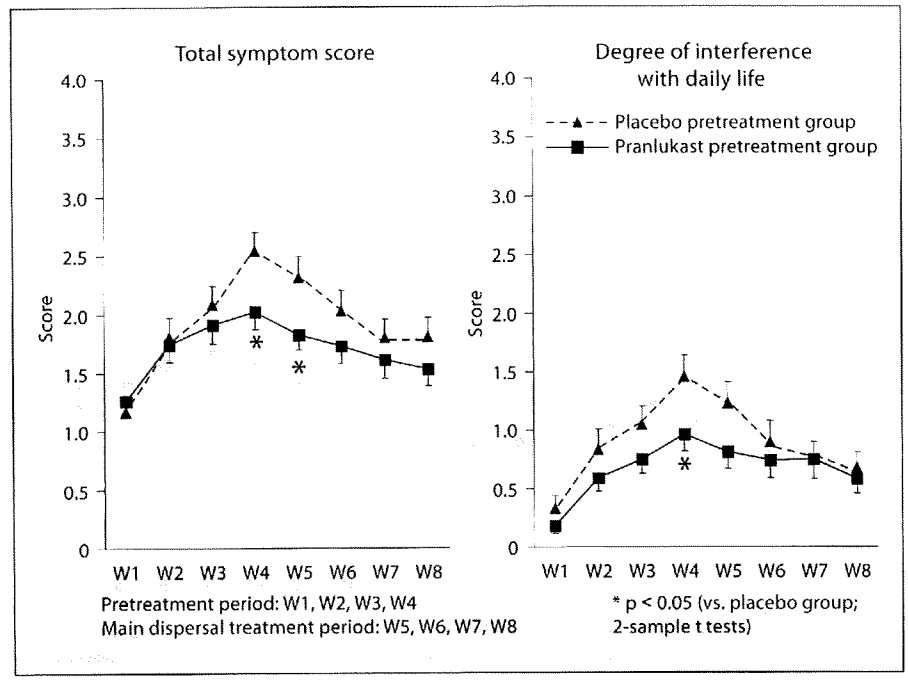

Fig. 4. Symptom scores and the degree of interference with daily life.

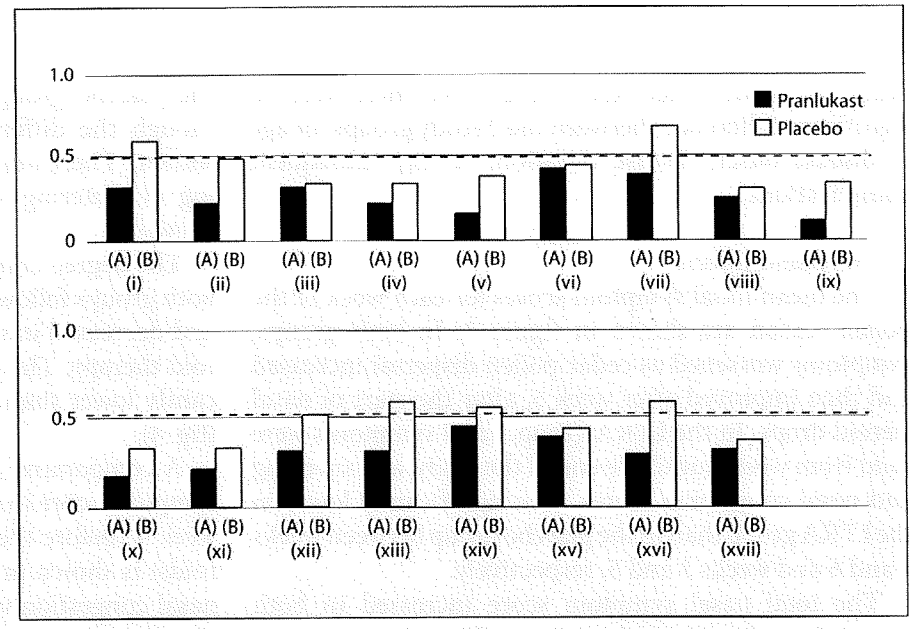

Fig. 5. QOL items at week 8 versus week 0. (i) = Reduced productivity at work/home/school; (ii) = poor mental concentration; (iii) = reduced thinking power; (iv) = impaired reading book/paper; (v) = reduced memory loss; (vi) = limitation of outdoor life (e.g., sports, picnic); (vii) = limitation of going out; (viii) = hesitation visiting friends or relatives; (ix) = reduced contact with friends or others by telephone or conversation; (x) = not an easy person to be around; (xi) = impaired sleeping; (xii) = tiredness; (xiii) = fatigue; (xiv) = frustration; (xv) = irritability; (xvi) = depression; (xvii) = unhappiness.

did not increase by 0.5 points at week 8 compared with week 0 (before pollen dispersal), scores for 7 items (interference with study, work and housework; poor concentration; interference with going out; malaise; fatigue; frustration; depressed feeling) in the placebo group still increased by ≥0.5 points at week 8.

These 17 QOL items were categorized into 6 domains (daily life; outdoor activities; social life; physical exercise; mental life; sleep) and scores were compared among domains (table 3). Mean QOL scores generally increased with cedar pollen dispersal when compared between week 4 and week 0, and scores increased by ≥0.5 points

Table 2. Improvement in nasal symptom score at week 8 compared with week 4

	Improved ≥1 point	Improved <1 point	χ^2 test[1]
Runny nose			
Pranlukast	16 (55.2)	13 (44.8)	0.977
Placebo	15 (55.6)	12 (44.4)	
Sneezing			
Pranlukast	16 (55.2)	13 (44.8)	0.803
Placebo	14 (51.9)	13 (48.1)	
Nasal congestion			
Pranlukast	20 (69.0)	9 (31.0)	0.034
Placebo	11 (40.7)	16 (59.3)	

Data are number of patients, with percentages in parentheses.

[1] The number of the patients who had an improved score of ≥1 point in the pranlukast-pretreated group was compared with that in the placebo-pretreated group.

Table 3. QOL score by JRQLQ (domains)

	Week	Pranlukast mean ± SE	Placebo mean ± SE
Versus week 0			
Usual daily activities	0	0.25 ± 0.08	0.13 ± 0.06
	4	0.97 ± 0.15***	0.87 ± 0.14***
	8	0.48 ± 0.10*	0.56 ± 0.14*
Outdoor activities	0	0.14 ± 0.05	0.19 ± 0.07
	4	1.02 ± 0.19***	1.20 ± 0.19***
	8	0.53 ± 0.14**	0.74 ± 0.18**
Social functioning	0	0.10 ± 0.04	0.06 ± 0.03
	4	0.69 ± 0.14***	0.53 ± 0.13***
	8	0.28 ± 0.09	0.38 ± 0.11**
Sleep problem	0	0.17 ± 0.07	0.19 ± 0.09
	4	0.59 ± 0.14**	0.81 ± 0.15***
	8	0.38 ± 0.10	0.52 ± 0.13*
General physical function	0	0.21 ± 0.08	0.13 ± 0.07
	4	1.00 ± 0.16***	0.96 ± 0.20***
	8	0.52 ± 0.12*	0.69 ± 0.18**
Emotional function	0	0.14 ± 0.05	0.06 ± 0.04
	4	0.78 ± 0.16***	0.74 ± 0.15***
	8	0.05 ± 0.14*	0.55 ± 0.15**
Overall QOL condition	0	1.07 ± 0.19	0.81 ± 0.13
	4	2.48 ± 0.15***	2.26 ± 0.19***
	8	1.45 ± 0.17	1.41 ± 0.16*
Versus week 4			
Usual daily activities	4	0.97 ± 0.15	0.87 ± 0.14
	8	0.48 ± 0.10***	0.56 ± 0.14*
Outdoor activities	4	1.02 ± 0.19	1.20 ± 0.19
	8	0.53 ± 0.14**	0.74 ± 0.18**
Social functioning	4	0.69 ± 0.14	0.53 ± 0.13
	8	0.28 ± 0.09***	0.38 ± 0.11
Sleep problem	4	0.59 ± 0.14	0.81 ± 0.15
	8	0.38 ± 0.10	0.52 ± 0.13
General physical function	4	1.00 ± 0.16	0.96 ± 0.20
	8	0.52 ± 0.12***	0.69 ± 0.18
Emotional function	4	0.78 ± 0.16	0.74 ± 0.15
	8	0.05 ± 0.14*	0.55 ± 0.15

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (2-sample t test).

for 5 of the 6 domains excluding sleeping problem in the LTRA group and for 5 domains excluding social functioning in the placebo group. After the start of nasal steroid therapy at week 5, scores in all 5 domains which had increased by ≥0.5 points at week 4 exhibited significant improvement at week 8 in the LTRA-treated group. The sleep problem and social functioning domains were not significantly aggravated at week 8 under Japanese cypress pollen dispersal when compared with week 0.

In contrast, in the placebo group, scores for 4 domains (social functioning; sleep problem; physical problems; emotional function) did not manifest significant improvement when evaluated during week 8 when compared with the scores observed at week 4 (before nasal steroid therapy). All domains exhibited a still significant increase at week 8 when compared with the scores observed at the beginning of the study (week 0).

Overall QOL condition scores also increased after pollen dispersal in both LTRA- and placebo-treated groups. After the use of nasal steroids, significant improvement was observed at week 8 in the LTRA-treated group but not in the placebo group compared with the scores at week 4.

Use of Concomitant Medications and Safety

Antihistamines and nasal vasoconstrictor drugs were used less frequently by the subjects in the LTRA group in the latter 2 weeks of the pretreatment period than in the placebo group; however, the differences were not significant (data not shown). The compliance for the use of LTRA and nasal steroids during the whole study period did not differ between the groups. However, an adverse event was reported by 1 patient in the LTRA group who experienced abdominal pain on day 16, but this resolved 2 days later and did not prevent continuation of study drug administration.

Discussion

In the present studies, the symptoms of allergic rhinitis increased predictably in the placebo group of subjects. However, many JRQLQ scores also worsened even in the LTRA group at the height of the pollen dispersal season. Therefore, pretreatment with LTRA alone did not appear to result in significant relief of nasal symptoms, and additional nasal steroid therapy in accordance with the standard guidelines was required to induce significant symptom relief [2, 4]. During the later phases of the cedar pollen dispersal season, symptoms and QOL scores exhibited improvement following the initiation of nasal steroid therapy in both LTRA and placebo-pretreated groups. However, differences between the groups in scores for sneezing and nasal congestion were still statistically significant. Subsequently, the symptoms in both groups improved and there were no significant differences in scores between the 2 groups. These findings are considered to reflect the effects of nasal steroid therapy. However, the degree of improvement in nasal congestion scores in week 8 at the end of the study compared with week 4 just before the concomitant therapy with nasal steroids was significantly higher in the LTRA-pretreated group than in the placebo-pretreated group.

QOL scores are considered to be more sensitive markers of clinical improvement than symptom scores derived from an allergy diary [21–23]. All QOL scores improved significantly in the LTRA group after the initiation of concomitant therapy with nasal steroids. The QOL items were categorized into 6 domains (daily life; outdoor activities; social life; physical exercise; mental life; sleep). Scores for the first 5 domains and the overall condition were significantly improved in the LTRA group after the initiation of concomitant nasal steroid therapy. Sleep was not significantly affected in the Japanese cypress pollen dispersal season. In contrast, in the placebo group, domain scores for social life, physical exercise, mental life and sleep (which was disturbed in the Japanese cypress pollen dispersal season in the placebo group) did not improve even after concomitant therapy with nasal steroid, and daily life and overall condition scores demonstrated delayed improvement compared with the LTRA group.

In Japan, pollen counts are typically measured using the gravimetric method with a Durham sampler, in contrast to Western countries in which a Burkard sampler is typically used. In a study in Chiba Prefecture in 2005, the amount of air-borne pollen counted with a Burkard sampler was about 12 times greater than that counted with a Durham sampler [24]. For Durham sampler measurements, a count of 1–10/cm^2/day is defined as low dispersal and >20/cm^2/day is considered high dispersal. In this study (2007), cedar pollen dispersal was detected by a Durham sampler at the beginning of February. The count was >20/cm^2/day on many days after 19 February and then returned to <10/cm^2/day after 10 March, after which dispersal ended. Japanese cypress pollen was detected at the end of February, had a count of >20/cm^2/day on many days after 19 March, with dispersal ending in early April.

Symptoms of allergic rhinitis are generally mild immediately after the start of the pollen dispersal season, but hypersensitivity-induced inflammation of the nasal mucosa is produced by repeated exposure to pollen. Such exposure results in enhanced expression of adhesion molecules, increased infiltration of the nasal mucosa by inflammatory cells, hyperpermeability of epithelial cells, and an increased neural sensory response [1, 2, 25]. Even in the LTRA group, many JRQLQ scores increased by ≥0.5 points at the height of the pollen dispersal season. With standard therapy using nasal steroids for severely affected patients, the QOL scores in the LTRA group were still lower than those in the placebo group. In the placebo group, nasal steroid therapy produced a smaller improvement in QOL scores.

The observations reported here and other earlier studies have suggested that LTRA are extremely safe and do not result in any major adverse effects, such as anticholinergic activity, local irritation or adrenal suppression. In this study, mild abdominal pain was reported by 1 patient, but no causal relationship with LTRA was detected.

Nasal steroids are generally very effective and provide a significant resolution of symptoms. Nasal steroids might be advantageous for early intervention; however, the compliance is sometimes poor, since many patients prefer to use oral medication, particularly in Japan [14, 15].

Although the number of patients enrolled in the study was limited and a comparative study with LTRA and steroids in a large scale will be needed to evaluate the effectiveness, based on the information summarized here, it is proposed that the use of LTRA is safe and might be appropriate for pretreatment before the appearance and establishment of clinical symptoms early in the course of the cedar pollen season.

Acknowledgements

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CD14 and *IL4R* gene polymorphisms modify the effect of day care attendance on serum IgE levels

To the Editor:

The cause of atopy is generally traced to the interplay of genetic and environmental factors.[1] Day care appears to be one of the most frequently investigated environmental factors. Although the results of studies investigating the association between day care attendance and atopy, as assessed by skin prick test responses, were inconsistent, all studies[2-4] measuring serum IgE levels have thus far shown a constant decreasing effect on serum IgE levels.

Among the genes that show a gene-environment interaction for the development of atopy or allergic diseases, the most frequently investigated is the *CD14* gene.[1] However, there is no report that investigates interaction of this gene and day care attendance. CD14 is a pattern-recognition receptor involved in the clearance of bacterial endotoxin and is also known as a receptor of respiratory syncytial virus. We investigated *CD14*−159C/T (rs2569190) and *CD14*−550C/T (rs5744455) polymorphisms in Japanese patients with severe respiratory syncytial virus–induced bronchiolitis and found that *CD14*−550C/T but not *CD14*−159C/T was significantly associated with the condition.[5]

The IL-4 receptor α gene *(IL4R)* is also one of the most frequently investigated genes and has been shown to be associated with atopy and atopic diseases.[6] The Ile50Val polymorphism (rs1805010) of the *IL4R* gene is a functional polymorphism and has been reported to be strongly associated with atopy and atopic asthma in the Japanese population. To date, only one study has reported the interaction of the *IL4R* Ile50Val polymorphism and day care attendance in the first year of life.[7] The result showed a

TABLE I. Characteristics of the subjects

Total no. of participants	473
Age (mo)	
Mean ± SD	111.1 ± 19.9
Range	76-147
Sex ratio (male:female)	1.00:1.01
Day care attendance before age 2 y (%)	14.5
Total IgE (IU/mL), mean ± SD	
Male	254 ± 340
Female	241 ± 469
Prevalence of atopy (%)	
Male	76.9
Female	68.0
Prevalence of allergic disorders (%)	
Asthma	
Male	14.1
Female	6.6
Atopic dermatitis	
Male	11.5
Female	9.7
Allergic rhinitis	
Male	42.1
Female	31.2
Food allergy	
Male	3.0
Female	3.4

significant gene-environment interaction for IFN-γ production at 1 year of age. However, it is not known whether this modified cytokine response affects the chance of having atopy or allergic diseases in the later period of life.

Here we report a relationship between serum total and specific IgE levels in Japanese elementary school children and day care attendance during earlier life. Our results suggest that day care attendance is associated with serum IgE levels, and this effect is modified by *CD14*−550C/T and *IL4R* Ile50Val polymorphisms. This is the first report that suggests an interaction between early-life day care attendance and genetic variations on IgE levels in later life.

Children attending an elementary school located in the central area of Chiba city (population of approximately 930,000) were recruited for this study. We first asked all (n = 843) children for participate in the survey. We then sent a detailed questionnaire to those who had a positive response (n = 582). Children with congenital heart diseases and lung diseases caused by immature birth were excluded. A total of 473 school children aged 6 to 12 years were enrolled. Blood samples were collected from 411 children on 2 separate days (July 3 and 12, 2006) for serum and DNA preparation. A complete set of information on total and 8 specific IgE levels, genotypes, and environmental factors was obtained from 375 children. All parents provided written informed consent. The study protocol was approved by the Ethics Committee of Chiba University Graduate School of Medicine.

The status of allergic diseases was evaluated by using questions based on the International Study of Asthma and Allergies in Childhood. We asked whether the child regularly attends a day care center where time is spent with other children at or before 2 years of age. For parents who responded yes to this question, the age of entry of their child to the day care center was obtained. The questionnaire also included the following items to assess possible confounding factors: number of siblings; number of older siblings; allergic diseases of parents and siblings (family history: scored as positive if parents, siblings, or both had any of 4 allergic diseases [asthma, allergic rhinitis, atopic eczema, and food allergy]); residential area (6 categories), type of house structure (5 categories), and floor type of bedroom (5 categories); yogurt/fermented food consumption; pet ownership; and smoking among family members.

Genotyping of the *CD14*−550C/T polymorphism was performed as described previously,[5] whereas that of the *IL4R* Ile50Val (rs1805010) polymorphism was carried out with the TaqMan allele-specific PCR method.[8] Primer sequences were as shown in this article's Online Repository at www.jacionline.org.

Table I shows the characteristics of the investigated population. The percentage of children who had regularly attended day care before 2 years of age was 14.5%. Atopy was defined as the presence of positive (≥0.35 IU/mL) specific IgE level against at least 1 of the 8 allergens. Although the prevalences of asthma, atopic dermatitis, and food allergy were compatible with those in a recent large study,[9] prevalences of allergic rhinitis and atopy were about 10 to 20 points higher, suggesting that children who had allergic rhinitis were more likely to attend this study.

Table II shows the association between day care attendance and serum IgE levels or atopy after being stratified with the *CD14*−550C/T genotype. Day care significantly decreased total IgE levels ($P = 9.7 \times 10^{-5}$), mite-specific IgE levels ($P = .0016$), and rate of atopy ($P = .00041$) in individuals with the C/T or T/T genotype, whereas the effect of day care was not observed in those with the C/C genotype. Numbers of children with the C/T+T/T genotype and those with the C/C genotype were similar, suggesting that the difference is not likely due to the statistical power for detecting association. Multivariate analyses with confounding factors were performed to evaluate the significance of this gene-environment interaction. The interaction between the *CD14*−550C/T polymorphism and day care was significant for $\log_{10}$(total IgE) ($P = .0046$), mite-specific IgE classes ($P = .00047$), and atopy ($P = .0097$) after adjusting for age, sex, family history, and number of siblings.

Table III shows the association between day care attendance and serum IgE levels or atopy after being stratified with the *IL4R* Val50Ile genotype. The effects of day care on total and some specific IgE levels were significant in Val/Ile heterozygotes but not in Val/Val or Ile/Ile homozygotes. In Val/Ile individuals day care significantly decreased total IgE levels ($P = .0012$), mite-specific ($P = .011$) and cedar pollen–specific ($P = .034$) IgE levels, and rate of atopy ($P = .018$). No such trend was observed in Val/Val or Ile/Ile individuals. The numbers of Val/Val and Val/Ile individuals were similar. It is therefore unlikely that the lack of significant association in Val/Val individuals was due to smaller statistical power for detecting association. When the significance of gene-environment interaction was assessed with the confounding factors, the interaction term between *IL4R* and day care attendance was significant for $\log_{10}$(total IgE) ($P = .019$) and mite-specific ($P = .0025$) and cedar pollen–specific ($P = .040$) IgE classes but not for atopy.

Total IgE levels in 4 genotype groups (group 1: *CD14* C/C, *IL4R* Ile/Ile+Val/Val; group 2: *CD14* C/C, *IL4R* Val/Ile; group 3: *CD14* C/T+T/T, *IL4R* Ile/Ile+Val/Val; and group 4: *CD14* C/T+T/T, *IL4R* Val/Ile) were compared to evaluate the combined effect of 2 polymorphisms on total IgE levels. Fig 1 shows the box

TABLE II. Effects of day care attendance on IgE levels when stratified by *CD14*–550C/T genotype

	C/C				C/T + T/T				
	Day care attendance		Effect size or odds ratio (95% CI)	*P* value	Day care attendance		Effect size or odds ratio (95% CI)	*P* value	Gene-environment interaction *P* value*
	No	Yes			No	Yes			
No. of subjects	169	22			157	28			
Log_{10}(total IgE)									
Mean	1.88	1.98	0.094 (−0.21 to 0.39)¶	0.54†	2.09	1.58	−0.50 (−0.26 to −0.76)¶	**9.7×10^{-5}†**	**.0046****
SD	0.77	0.76			0.63	0.51			
Specific IgE (positive‡ rate)									
Mite	0.49	0.59	1.50 (0.61 to 3.69)#	.51§	0.61	0.32	0.30 (0.13 to 0.71)#	**.0016§**	**.00047††**
Cedar pollen	0.45	0.46	1.02 (0.42 to 2.45)#	.92§	0.57	0.32	0.35 (0.15 to 0.83)#	**.032§**	.116††
Atopy (rate)	0.77	0.68	1.60 (0.56 to 4.55)#	.38‖	0.81	0.50	0.24 (0.10 to 0.55)#	**.00041‖**	**.0097‡‡**

Boldface indicates statistically significant values.
*Adjusted for age, sex, number of siblings, and family history.
†Analysis of variance for log_{10}(total IgE [in international units per milliliter]).
‡Class ≥1 (≥0.35 IU/mL).
§Kruskal-Wallis test for IgE value (in international units per milliliter).
‖χ^2 Test of independence.
¶Effect size.
#Odds ratio.
**General liner model.
††Generalized linear model (Poisson distribution, log link function).
‡‡Logistic regression.

TABLE III. Effects of day care attendance on IgE levels when stratified by *IL4R* Val50Ile genotype

	Val/Val				Val/Ile				Ile/Ile				
	Day care attendance		Effect size or odds ratio (95% CI)	*P* value	Day care attendance		Effect size or odds ratio (95% CI)	*P* value	Day care attendance		Effect size of odds ratio (95% CI)	*P* value	Gene-environment interaction *P* value*
	No	Yes			No	Yes			No	Yes			
No. of subjects	125	18			152	27			49	5			
Log_{10}(total IgE)													
Mean	1.94	1.91	−0.058 (−0.38 to 0.27)¶	.72†	1.88	1.55	−0.44 (−0.71 to −0.18)¶	**.0012†**	1.99	2.32	0.33 (−0.31 to 0.97)¶	.12†	**.019****
SD	0.64	0.72			0.57	0.56			0.69	0.52			
Specific IgE (positive‡ rate)													
Mite	0.57	0.56	0.95 (0.35 to 2.57)#	.51§	0.52	0.30	0.39 (0.16 to 0.94)#	**.011§**	0.59	0.80	2.76 (0.29 to 26.5)#	.36§	**.0025††**
Cedar pollen	0.50	0.50	1.01 (0.38 to 2.73)#	.93§	0.51	0.30	0.41 (0.17 to 0.99)#	**.034§**	0.55	0.40	0.54 (0.083 to 3.54)#	.91§	**.040††**
Atopy (rate)	0.74	0.72	0.93 (0.31 to 2.82)#	.90‖	0.74	0.52	0.37 (0.16 to 0.86)#	**.018‖**	0.76	0.80	1.30 (0.13 to 12.8)#	.82‖	.118‡‡

Boldface indicates statistically significant values.
*Adjusted for age, sex, number of siblings, and family history.
†Analysis of variance for log_{10}(total IgE [in international units per milliliter]).
‡Class ≥1 (≥0.35 IU/mL).
§Kruskal-Wallis test for IgE value (in international units per milliliter).
‖χ^2 Test of independence.
¶Effect size.
#Odds ratio.
**General liner model.
††Generalized linear model (Poisson distribution, log link function).
‡‡Logistic regression.

plot of log_{10}(total IgE) in 4 genotype groups. Among children who attended day care compared with group 1, the mean log_{10} (total IgE) values of groups 2, 3, and 4 decreased by 0.41, 0.35, and 0.69, respectively. This magnitude of change suggests that the effects of *CD14* and *IL4R* were additive. The children in group 4 showed significantly (P = .0046) lower total IgE levels than those in group 1. On the other hand, among children who did not attend day care, the log_{10}(total IgE) levels of children in groups 3 (P = .031) and 4 (P = .036) were significantly higher than those of children in group 1. The *CD14* C/T and T/T genotypes appeared to show the opposite effect on the serum total IgE level in children who did not attend day care compared

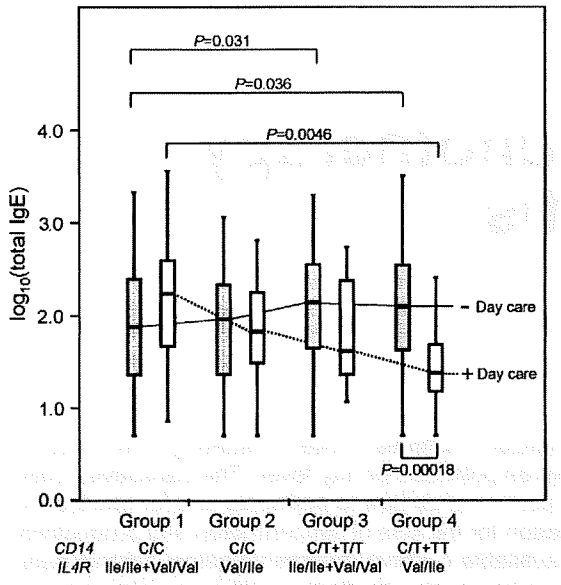

FIG 1. Total IgE levels in 4 groups of children classified based on a combination of *IL4R* and *CD14* genotypes. Box plot of $\log_{10}$(total IgE) values is shown for children who attended day care *(+Day care)* and for those who did not *(−Day care)*. Results are presented as medians and interquartile ranges. Only significant *P* values (<.05) are shown.

with those who did attend day care. When we examined the effect of day care in each genotype group, the effect was not sufficiently large to show a significant change in IgE level in groups 2 and 3, in which individuals had only 1 IgE level–decreasing genotype. However, in group 4, in which individuals had 2 IgE level–decreasing genotypes, the effect was sufficiently large to show a significant difference ($P = .00018$). Significance of interaction between the *CD14* and *IL4R* genotypes was also evaluated by using general linear models in which age, sex, family history, number of siblings, and day care were included as variables. The interaction term of the 2 genes was not significant, suggesting an independent effect of the *CD14* and *IL4R* genes.

The interaction of the *CD14* gene with day care attendance suggests that the mechanism of the effect of day care involves at least in part a response to infection, environmental endotoxin exposure, or both. The interaction of the *IL4R* gene with day care attendance suggests that the mechanism also involves those related to T_H2 cell proliferation and IgE production. These results suggest that the complex nature of mechanisms underlies the effect of day care attendance on serum IgE levels.

Environmental factors investigated in the present study were determined based on a questionnaire on past day care attendance, and therefore recall bias can be a potential problem. The number of subjects investigated in this study was not so large and might be the acceptable minimum for investigating gene-environment interactions. The subjects evaluated were children who attended a single school and lived in a medium-populated city, thus representing those living in rather small regional environments in Japan. Nevertheless, these characteristics of the present sample might have contributed to minimizing the variances of background and outcome parameters and might have resulted in the positive findings obtained from a relatively small number of subjects. It is necessary to perform a cohort study to follow children with or without day care attendance until they reach school age to validate the current observations.

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REVIEW ARTICLE

Antigen-Specific Immunotherapy against Allergic Rhinitis: The State of the Art

Takashi Fujimura[1,2] and Yoshitaka Okamoto[1]

ABSTRACT
Allergic rhinitis is the most prevalent type I allergy in industrialized countries. Pollen scattering from trees or grasses often induces seasonal allergic rhinitis, which is known as pollinosis or hay fever. The causative pollen differs across different areas and times of the year. Impaired performance due to pollinosis and/or medication used for treating pollinosis is considered to be an important reason for the loss of concentration and productivity in the workplace. Antigen-specific immunotherapy is an only available curative treatment against allergic rhinitis. Subcutaneous injection of allergens with or without adjuvant has been commonly used as an immunotherapy; however, recently, sublingual administration has come to be considered a safer and convenient alternative administration route of allergens. In this review, we focus on the safety and protocol of subcutaneous and sublingual immunotherapy against seasonal allergic rhinitis. We also describe an approach to selecting allergens for the vaccine so as to avoid secondary sensitization and adverse events. The biomarkers and therapeutic mechanisms for immunotherapy are not fully understood. We discuss the therapeutic biomarkers that are correlated with the improvement of clinical symptoms brought about by immunotherapy as well as the involvement of Tr1 and regulatory T cells in the therapeutic mechanisms. Finally, we focus on the current immunotherapeutic approach to treating Japanese cedar pollinosis, the most prevalent pollinosis in Japan, including sublingual immunotherapy with standardized extract, a transgenic rice-based edible vaccine, and an immunoregulatory liposome encapsulating recombinant fusion protein.

KEY WORDS
allergic rhinitis, biomarker, immunotherapy, pollinosis, regulatory T cell

INTRODUCTION

Allergic rhinitis is the most prevalent type I allergy, and pollen grains are one of the most common causes of respiratory allergies. In western Europe, the prevalence of clinically confirmable allergic rhinitis was estimated to be 23%, with more than 50% of the allergic subjects possessing specific IgE against grass pollen.[1] In Japan, the prevalence of allergic rhinitis was estimated to be 39.4% and that of pollinosis was 29.8%.[2]

Pollinosis is induced by the invasion of pollen grains onto the ocular and nasal mucosa. Pollen grains easily access internal binding sites on contact with the aqueous phases of nasal and ocular mucosal membranes. After pollens are hydrated on aqueous membranes, they swell, rupture, and release their cytoplasmic components. It has been reported that grass pollen grains rupture in water and release large amounts of respirable particles, such as starch granules containing allergens.[3] Although pollinosis patients have a low rate of asthma attacks during pollen season, the attacks that do occur may be attributable to these respirable particles bearing allergens from pollen grains.[4] Pollen grains release not only allergen-bearing particles but also immunomodulatory mediators such as pollen-associated lipid mediators (PALMs) and NADPH oxidases. Proinflammatory PALMs such as leukotriene B4-like substances attract and activate human peripheral blood eosino-

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phils and polymorphonuclear granulocytes from both allergic and non-allergic donors.[5,6] Immunomodulatory PALMs, such as phytoprastanes, inhibit IL12 production in dendritic cells and Th1-type cytokine production in antigen-specific T cells, while inducing antigen-specific Th2 responses.[7] NADPH oxidase rapidly increases the level of reactive oxygen species (ROS) in lung epithelium and induces neutrophil recruitment to the airway independent of the adaptive immune responses.[8,9] These reports strongly suggest that pollen grains themselves act primarily as adjuvants to induce pollen-antigen-specific Th2 responses and to enhance inflammatory processes during the elicitation phase of allergic responses.

The most common treatments against pollinosis are medications like antihistamines, leukotriene inhibitors, and corticosteroids. However, these treatments are not curative and sometimes induce impaired performance as a results of their side effects.[10,11] Antigen-specific immunotherapy can change the natural course of allergic rhinitis and is recognized as a curative treatment against type I allergy without impaired performance. In this century, since the first report on subcutaneous immunotherapy (SCIT), SCIT has been developed and improved and has become safer and more effective.[12,13] Recently, sublingual immunotherapy (SLIT) has been developed and has become a safer and more beneficial immunotherapy for patients.

This review focuses on the recent approach of using antigen-specific immunotherapy to treat allergic rhinitis, and focuses especially on the use of SLIT against pollinosis using standardized extract or recombinant allergens. We also discuss the therapeutic mechanisms and therapeutic biomarkers for SLIT. Finally, we discuss the recent immunotherapeutic approach to treat Japanese cedar (*Cryptomeria japonica*) pollinosis, which is the most common pollinosis in Japan.

ANTIGENS FOR IMMUNOTHERAPY

For immunotherapy, extracts from an allergen source, i.e. pollen extract, are widely used after the concentration of their major allergen is adjusted so as to be standardized. To standardize such extracts, it is important to analyze their component allergens and establish a quantification system for major allergens.[14] The World Allergy Organization (WAO) recommends that standardized vaccines be used for immunotherapy if they are available.[15] However, the protocols and methods for the standardization of allergen extract are different among different suppliers, which use their own in-house reference materials and their own unique allergen units. This made it difficult to compare the therapeutic effects and safety among clinical trials involving different products. It has been proposed that vaccines be standardized using a protocol based on mass units of major allergens and that the active ingredients of the treatment be quantified. The CREATE project has been working to select major allergens for use in the standardization of vaccines and to establish a quantification system and recombinant allergens for the standardization.[16]

To improve the safety and clinical therapeutic effects of a vaccine, the selection of allergens for vaccination is an important issue. Extract from pollen may contain many allergens that cross-react with those from fruit, vegetables, and latex. These allergens may cause minor local side effects, especially in SLIT, among patients who suffer from oral allergies and/or latex-fruit syndrome. Latex-fruit syndrome sometimes induces severe systematic reactions such as anaphylactic shock in response to natural rubber and some latex fruits.[17] The cross-reactive allergens may have to be removed from vaccines in order to avoid severe systematic adverse reactions caused by cross-reactivity with latex allergens for safer SLIT. For the elucidation of reactive allergens, protein microarray techniques have recently been applied to allergy diagnosis. Microarray-chip technology using a glass slide with the immobilization of large numbers of proteins on the surface enable us to simultaneously test IgE-binding reactivity against large numbers of allergens from various sources.[18,19] This diagnostic technique is applicable to the diagnosis of allergens from a single allergen source. This component-resolved diagnosis is a powerful tool for selecting components of allergens for immunotherapy vaccines and may improve the safety and clinical therapeutic efficacy of the vaccines in comparison to traditional immunotherapy using crude extract.[20] Such an allergen diagnosis enables us to choose only IgE-binding allergens that are individually sensitized for antigen-specific immunotherapy. This approach, in which only sensitized allergens are used for immunotherapy, avoids secondary additional sensitization against nonreactive proteins that can occur with the use of crude extracts or a mixture of allergens (Fig. 1).

Recombinant technology has been used to construct vaccines for immunotherapy.[21] Immunotherapy clinical trials were performed using a mixture of five recombinant grass allergens (rPhl p 1, rPhl p 2, rPhl p 5a, rPhl p 5b, and rPhl p 6), and the results suggested that a recombinant allergen vaccine can be an effective and safe treatment to ameliorate the symptoms of allergic rhinitis.[22] Immunotherapy using recombinant Bet v 1 was also recently reported to show clinical efficacy, and its therapeutic effects were comparable with those obtained using native Bet v 1 against birch pollen allergy.[23]

Vaccines using allergoids and modified allergens, such as T cell-epitopes, pathogen-related molecular pattern molecule-conjugated allergens, and others, are under development, and some of them are considered to be promising for use as therapeutic vaccines.[13,24]

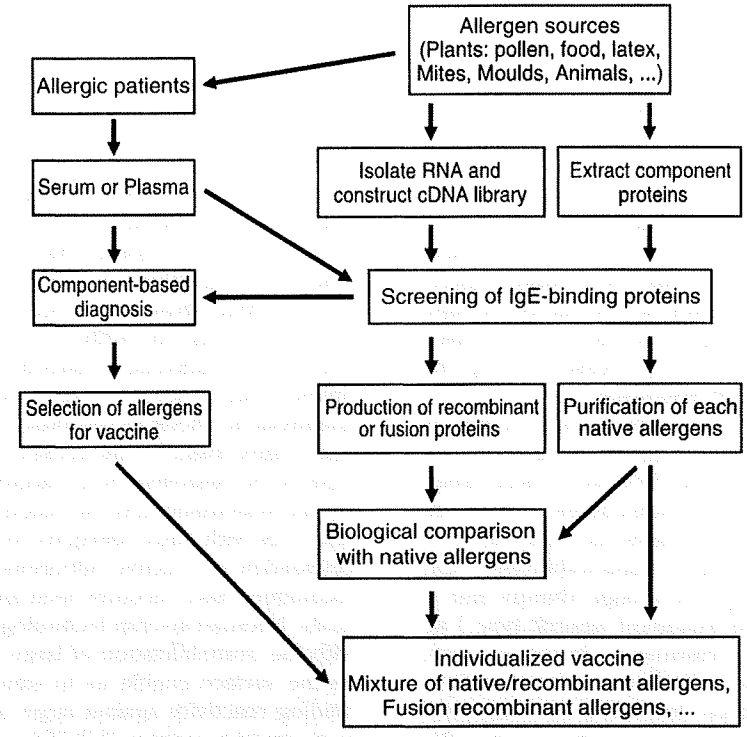

Fig. 1 Schematic procedure of the steps involved in the identification and development of an individualized vaccine using only sensitized antigens for immunotherapy. To identify component allergens which have the capacity to react with serum IgE from allergic patients, it is important to establish individualized vaccines to avoid secondary sensitization. Allergens with which an individual patient reacted can be elucidated by a component-based diagnosis, and an individualized vaccine can be established using a mixture of the purified native or the standardized recombinant allergens to which the patient is sensitized.

ROUTE OF VACCINE ADMINISTRATION FOR IMMUNOTHERAPY AND ITS SAFETY

Immunotherapy vaccines against allergies were originally injected subcutaneously without an adjuvant.[12] However, subcutaneous injection of allergens often induces severe adverse reactions like local allergic reactions, urticaria, asthma, and frequent anaphylaxis. To increase the safety and therapeutic efficacy of immunotherapy vaccines, aqueous allergen extracts absorbed into adjuvants such as aluminum hydroxide have been used in SCIT.[25] Pretreatment with antihistamine or anti-IgE antibody has been used to prevent the adverse events that can be induced after subcutaneous vaccine injection, and the pretreatments also enhance the therapeutic efficacy of SCIT.[26,27]

In this decade, SLIT has been developed as a safer method for immunotherapy and has been used with increasing frequency, especially in Europe and the US. SLIT is noted to be a very safe method without fetal adverse reactions. In most cases, adverse reactions to SLIT have been mild local reactions such as oral pruritus, edema of the mouth, throat irritation, and sneezing.[28] However, a few cases of anaphylaxis have been reported after SLIT using a crude or standardized vaccine.[29-33] These reports suggest that SLIT is not always safe for patients, especially those with severe asthma or who have experienced severe adverse reactions to SCIT. It has been recommended that the first dose of the vaccine is to be administered in a doctor's office under observation.[32]

The administration regimens for SLIT, including dosing, the build-up phase, duration of the treatment, and frequency of the maintenance dose, differ greatly among the clinical trials.[34] The sublingual and supralingual administration methods of oral drops were evaluated by a double-blind, placebo-controlled study using mixed standardized extract in patients allergic to grass pollen. In this report, sublingual administration significantly reduced the nasal, ocular, and bronchial symptoms, as well as the intake of symptom-reducing drugs compared to the placebo. Supralin-

Table 1 Comparison between SLIT and SCIT

	SLIT	SCIT
Administration	Sublingual spitting or sublingual swallowing	Subcutaneous injection with or without adjuvant
Pre-treatment	None	Medication or anti-IgE
Build-up phase	A few weeks, one day for rush protocol, or no up-dosing phase	A few weeks or a few days for rush protocol
Vaccination	Once daily or a few times weekly	A few times weekly or monthly
Adverse event	Local mild reaction in most cases, a few reports of fetal adverse reactions	Sometimes induces fetal adverse reactions

gual treatment also attenuated the symptoms and symptom-reducing drugs intake; however, only the nasal symptom score showed a significant reduction compared to the placebo-control group.[35] Thus, holding the vaccine under the tongue may be an important way to achieve better therapeutic effects with SLIT.

Vaccines for SLIT can also be delivered by two methods: sublingual spitting, in which the vaccine is spat out after being held under the tongue, and sublingual swallowing, in which the vaccine is swallowed after being kept under the tongue. In studies using radiolabeled allergens, most of the allergens remained in the mouth after the vaccine was spat out. However, plasma radioactivity began to increase only after swallowing.[36-38] The author concluded that contact between the allergens and the oral mucosa is a crucial step in the mechanisms of SLIT, and suggested that the more appropriate and advantageous way to administer the allergen sublingually is via the sublingual swallowing procedure.[38]

It has been recommended that the administration of SLIT vaccine be started at least 8 weeks before pollen season for better therapeutic effects.[39] However, an ultra-rush scheme of SLIT treatment for children allergic to grass pollen was reported to significantly improve the symptoms and the medication score compared to the placebo group. In this 2-year randomized, double-blind, placebo-control trial, the authors administered standardized extract of five grass pollen (*Dactylis glomerata*, *Anthoxanthum odoratum*, *Lolium perenne*, *Poa prantensis*, and *Phleum pretense*) beginning 2 weeks before the pollen season started with one day for ultra-rush induction, and followed by daily treatment (120 IR, 10 μg major allergen) for 6 months. It has been reported that SLIT significantly improved the asthma symptom score and reduced the nasal symptom score and the use of rescue medication score compared to the placebo group.[40] The starting point and duration of treatment varied among the clinical trials, and the best procedure for administration remains unclear.[41] (Table 1)

As a novel route to enhance the therapeutic efficacy of the vaccine, direct intralymphatic injection was proposed for the administration of peptide vaccine against viral infection and tumor in the mouse. This paper reported that the direct administration of peptide vaccine into a lymph node induced enhanced immunogenicity compared to subcutaneous and intradermal vaccination.[42] This novel technique was recently applied to patients with hay fever in an open-label, randomized control trial.[43] The authors injected 1,000 SQ-U of aluminum hydroxide-adsorbed grass pollen extract into a superficial inguinal lymph node under ultrasonic guidance. Three intralymphatic injections over 2 months resulted in long-lasting tolerance with the amelioration of hay favor symptoms, reduced skin prick test reactivity, and decreased serum allergen-specific IgE comparable with conventional SCIT. Furthermore, the author reported that there were fewer adverse events than in SCIT, even without premedication with antihistamines, and the injection was less painful than venous puncture.[43] Further clinical trials with a larger population are needed to evaluate the safety, therapeutic efficacy, and duration of tolerance of this treatment.

BIOMARKERS FOR SLIT

The therapeutic effects obtained by antigen-specific immunotherapy are commonly judged on the basis of clinical symptoms according to quality-of-life (QOL) score, symptom diary, and symptom-reducing drugs intake. The biomarkers correlated with the therapeutic effects are still controversial, especially for SLIT.

Antigen-specific IgG4 is considered to be a biomarker for antigen-specific immunotherapy; however, the correlation between the induction of IgG4 production and clinical symptoms is controversial.[44] In a report about the use of SLIT against timothy pollinosis, antigen-specific IgG4 was significantly upregulated in the SLIT group compared to the placebo group, and the authors concluded that the upregulation of IgG4 was correlated with the improvement of symptoms compared with the previous year. However, the clinical score and medication score were not significantly different between the SLIT group and the placebo group.[45] A recent study of dairy administration of grass allergen tablets showed dose-dependent efficacy of the SLIT and the induction of blocking IgG. This report showed that the administration of 75,000 SQ-T (15 μg Phl p 5) dose significantly reduced the symptom and medication

scores, and up-regulated specific IgG; however, a 2,500 SQ-T (0.5 μg Phl p 5) dose did not result in amelioration of the symptom and medication scores nor in the induction of IgG.[46] We previously reported that specific IgG4 was significantly increased in pollen season concomitant with improvement of the symptom medication score in the SLIT group compared to the placebo group.[47] The disagreement in results related to the induction of blocking IgG or IgG4 and the improvement of clinical symptoms may depend on the dose and/or the method of administration of the SLIT vaccine.

Other serological parameters have been recently reported to be useful as therapeutic biomarkers for SLIT. A 3-month course of pre-seasonal treatment of patients with grass pollen allergic rhinitis induced a reduction of the serum level of soluble human leukocyte antigen (sHLA)-G. The authors reported a significant relationship among the decrease of the sHLA-G serum level, the increase of interferon (IFN)-γ producing cells, and the decrease of sHLA-A, -B, and -C after SLIT.[48] Furthermore, the changes of serum sHLA levels were significantly correlated with the clinical symptom score measured using a visual analogue scale (VAS) after SLIT.[49] In this preliminary open-labeled study, the authors suggested that sHLA molecules might be considered as possible biomarkers of the response to SLIT.

Recently, two reports investigated the change of serum reptin levels after SLIT. Leptin is primarily produced by adipocytes and has been reported to protect T lymphocytes from apoptosis, regulate T cell activation, and up-regulate adhesion molecules in endothelial cells.[50] Furthermore, leptin was reported to modulate the hyporesponsiveness and proliferation of human naturally occurring $Foxp3^+CD25^+CD4^+$ regulatory T (nTreg) cells.[51] After a 3-month course of SLIT against pollinosis, serum leptin levels were reported to significantly correlate with symptom severity as assessed by VAS of nasal symptoms in women, the number of peripheral eosinophils in men, the allergen threshold dose for allergen-specific nasal challenge in both men and women, and the medication score in women. This 3-month course of SLIT showed a tendency to increase serum leptin levels compared to the levels before the SLIT, albeit the increase was not significant.[52] After a 2-year course of SLIT, the serum leptin level was significantly increased in men.[53] The relationship between the up-regulation of leptin by SLIT and clinical symptoms remains unclear; however, the difference of the clinical therapeutic efficacy may depend on gender and the presence or absence of obesity.

The reduction of antigen-specific Th2 responses is considered to be an important biomarker for antigen-specific immunotherapy. The increase in the size of the specific Th2 clone, which produces IL4 after being stimulated with Cry j 1 (a major allergen of the Japanese cedar pollen), after pollen season was reported to be significantly reduced in the SLIT group compared with the placebo group in a double-blind, placebo-controlled study of Japanese cedar pollinosis. The increase of specific IL5-producing cells after pollen season was also reduced in the SLIT group, but the reduction was not statistically significant.[47] It has also been reported that after a 2-year course of SCIT against Japanese cedar pollinosis, B and T lymphocyte attenuator (BTLA) expression on $CD4^+$ T cells was down-regulated in untreated patients after Cry j 1 stimulation and up-regulated in SCIT-treated patients. Furthermore, the change of BTLA expression was negatively correlated with IL5 production. The authors concluded that BTLA-mediated coinhibition of IL5 production may contribute to the regulation of allergen-specific T cell responses by antigen-specific immunotherapy.[54]

The therapeutic biomarkers of SLIT in children also remain unclear. In a study of the administration of the SLIT treatment to children with seasonal allergic rhinoconjunctivitis to grass pollen, the authors reported that a 2-year course of SLIT using a standardized 5-grass mixture (1.5 μg/week) did not alter the systemic immunologic reaction of IL4, IL5, and IFN-γ cytokine production, nor the proliferation of PBMC after stimulation with allergens in the SLIT group compared to the placebo group, although a positive effect on rescue medication use was achieved by SLIT treatment.[55] However, another study reported the up-regulation of mRNA expression in PBMC during SLIT in children using SQ-standardized tree pollen extracts. The authors reported that after the stimulation of PBMC with allergen *in vitro*, the mRNA expression of signaling lymphocytic activation molecule (SLAM) was significantly increased from baseline after 1 year in the SLIT group receiving a high-dose (weekly dose of 200,000 SQ-U) treatment. This up-regulation was reported to be correlated with IL10 and transforming growth factor-β (TGF-β) mRNA expression. The IL18 mRNA expression was also increased in the high-dose group over a 1-year treatment compared to the placebo group and was reported to be inversely correlated with the late-phase skin reaction after the second study year. The authors reported that this up-regulation of SLAM and IL18 mRNA expression suggested the down-regulation of Th2-type inflammatory responses by increased Th1-type responses.[56] Another study of SLIT in children using SQ-standardized tree pollen extract (weekly dose of 200,000 SQ-T, 30 μg major allergen containing Bet v 1, Aln g 1, and Cor a 1) reported that specific allergen-induced Foxp3 mRNA expression after a 2-year course of SLIT treatment was significantly increased in PBMCs compared to the placebo group and compared to the level before treatment. Changes in allergen-induced Foxp3 expression that significantly correlated with IL10 mRNA expression

were reported in the whole study group, including the low-dose (weekly dose of 24,000 SQ-T) group and the placebo group, after 1- and 2-year courses of treatment, and correlated with TGF-β1 mRNA after 1 year of treatment. Furthermore, IL17A mRNA expression was significantly correlated with symptom-medication score (SMS) in the whole study group and especially in the high-dose treated group. The authors concluded that IL17 expression may be associated with a poor therapeutic outcome of SLIT.[57]

MECHANISMS OF ANTIGEN-SPECIFIC IMMUNOTHERAPY

Numerous data showing that antigen-specific Th2-type responses are down-regulated and, in contrast, Th1-type and/or regulatory T cell (Treg) responses are up-regulated by immunotherapy have been accumulated. The imbalance of the population among the antigen-specific Th1, dominant Th2, and Treg is considered to induce sensitization and subsequent allergic inflammation in response to invading allergens, and immunotherapy may correct the imbalance of these cells. Actually, the high frequency of IL4-secreting Th2 cells was reported in allergic individuals, as was, in contrast, the dominance of IL10-secreting Tr1 cells in healthy subjects.[58] These authors suggested that the balance between allergen-specific Tr1 cells and Th2 cells causes the development of the allergy.

IL10-producing regulatory cells are considered to play a crucial role in clinical therapeutic mechanisms in immunotherapy. In a study of SCIT using house dust mite (HDM) extract in patients allergic to HDM, SCIT induced the suppression of PBMC proliferation and the suppression of IFN-γ, IL5, and IL13 production in PBMC stimulated with Der p 1 (a major allergen of HDM) at 70 days after treatment compared to the levels before treatment. In contrast to the suppression of Th1 and Th2 cytokines, the production of both IL10 and TGF-β was significantly increased. The report also showed that the suppression of proliferation was dependent on IL10 and TGF-β and that the source of IL10 is $CD25^+CD4^+$ T cells.[59] It has also been reported that IL10 production was induced by SLIT against HDM. The authors also reported the suppression of the proliferation of PBMC stimulated with extract of mite (*Dermatophagoids farinae*) and the increase of IL10 production compared to non-treated subjects.[60] The IL10 production after 3 years of SLIT treatment was significantly correlated with the improvement of clinical symptoms as assessed by forced expiratory flow between 25% and 75% (FEF_{25-75}).[61]

In a report about the use of SLIT to treat birch pollinosis, the authors investigated the antigen-specific proliferation and mRNA levels of cytokines and Foxp3. They reported that 4 weeks of SLIT induced a reduction in Bet v 1-specific proliferation and induced mRNA expression of IL10 and Foxp3 in $CD3^+$ cells compared to the levels before SLIT. These up-regulations of IL10 and Foxp3 mRNA expression were not seen after 52 weeks after SLIT; however, IFN-γ mRNA expression was significantly induced at 52 weeks after SLIT. The reduced Bet v 1-specific proliferation was significant after both 4 and 52 weeks, and this down-regulation was dependent on IL10 at 4 weeks. It has also been reported that neither TGF-β levels nor cell-cell contact-mediated suppression of $CD25^+CD4^+$ cells were changed during the course of SLIT.[62] Another report shows the significant reduction of IL5 mRNA expression and increased IL10 expression compared to the placebo group after 1 and 2 years of SLIT at a weekly dose of 200,000 SQ-U (30 μg major allergen) in children with tree pollinosis. It has been reported that TGF-β expression remained low after 1 and 2 years of SLIT; however, TGF-β expression was inversely correlated with IL5 and positively correlated with IL10 expression after 1 year of SLIT.[63]

In addition to IL10-secreting Tr1 cells, $Foxp3^+$ Treg cells are also considered to play a crucial role in the therapeutic effects achieved by immunotherapy (Fig. 2). It has been reported that 2 years of SCIT against hay fever significantly induced an increase in the number of $Foxp3^+CD25^+$ and $Foxp3^+CD4^+$ cells in the nasal mucosa compared to the number before SCIT and the number in untreated patients out of season. Twenty per cent of $CD3^+CD25^+$ cells were reported to also be Foxp3-positive, and 18% of $CD3^+$IL10-expressing cells were Foxp3-positive in the nasal mucosa after immunotherapy. This report suggested that the increase of $Foxp3^+CD25^+CD3^+$ cells in the nasal mucosa was associated with the clinical efficacy and suppression of seasonal allergic inflammation. This report also suggested the involvement of different types of regulatory T cells, namely IL10-secreting Tr1 cells and adaptive or induced Foxp3-positive Treg, in the therapeutic mechanisms of immunotherapy.[64] The involvement of Treg cells in immunotherapy was also reported in SCIT against hymenoptera venom allergy. In this report, the authors showed that the numbers of peripheral Treg cells defined as $Foxp3^+CD25^{bright}CD4^+$ T cells were significantly increased by venom immunotherapy, and the increase of circulating Treg cells was significantly correlated with the venom specific IgG4/IgE ratio.[65]

Antigen-specific Tr1 and Treg cells are considered to be involved not only in the suppression of Th2 cells but also, directly or indirectly, in the suppression of peripheral allergic inflammation[24] (Fig. 3). It has been reported that $CD25^+CD4^+$ Treg cells, more than 90% of which are $Foxp3^+$, directly inhibited the FcεR1-dependent mast cell degranulation after crosslinking of IgE, and this inhibition was dependent on cell-cell contact involving OX40-OX40L interactions between Treg and mast cells in the mouse.[66] Furthermore, al-

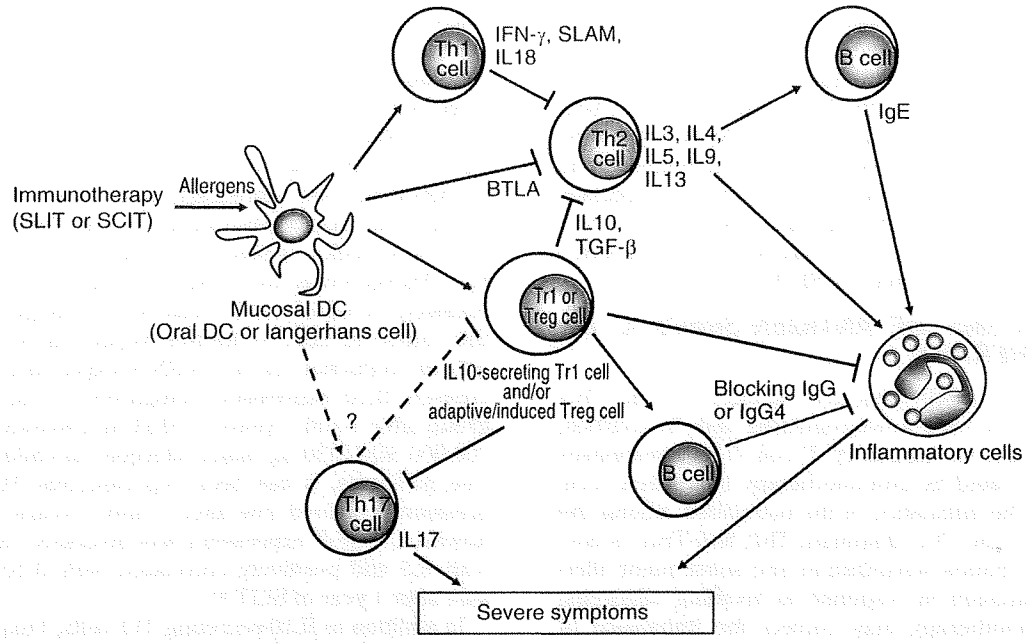

Fig. 2 T cells in antigen-specific immunotherapy. Antigen-specific immunotherapy induces regulatory T cells and Th1 cells via antigen-presentation by mucosal dendritic cells (DC). Th17 cells may be induced in a non-responder population by immunotherapy. The induced Th1 cells and/or regulatory T cells down-regulate the activation of Th2 cells and subsequently the activation of inflammatory cells such as eosinophils and mast cells. The regulatory T cells also activate B cells to produce blocking IgG or IgG4, and the blocking antibody inhibits binding between allergen and surface IgE on inflammatory cells to prevent the secretion of inflammatory chemical mediators.

lergic human eosinophils in peripheral blood and chronically inflamed nasal tissues were reported to express CD40, and the cross-linking of CD40 and CD40L enhanced the survival of eosinophils and induced the release of granulocyte/macrophage colony-stimulating factor (GM-CSF). In this report, IL10 down-regulated the constitutive expression of CD40 mRNA expression in eosinophils.[67] The induction of IL10-producing Tr1 or Treg cells in the nasal mucosa may play an important role in the reduction of nasal symptoms via cross-talk down-regulation of mast cells and eosinophils.

In a reports on the rush protocol of SCIT against Japanese cedar pollinosis using standardized pollen extract, the percentage of $CD203c^{high}$ cells in $CD3^{-}$ $CRTH2^{+}$ basophils after allergen stimulation was reported to be down-regulated after rush immunotherapy without a decrease of the serum specific IgE titer. Furthermore, the percentage of $CD203c^{high}$ on basophils after *in vitro* stimulation was reported to be significantly correlated with symptom score.[68] The mechanisms which attenuate the sensitivity of peripheral basophils without a change in serum specific IgE remain unclear; however, this attenuation may be partially due to the up-regulation of inhibitory blocking antibody on the surface of basophils.

ANTIGEN-SPECIFIC IMMUNOTHERAPY AGAINST JAPANESE CEDAR POLLINOSIS

In Japan, Japanese cedar pollinosis is one of the most prevalent types of seasonal allergic rhinitis, with a prevalence estimated to be 26.5%.[2] Two clinical trials described the therapeutic effects of SLIT against Japanese cedar pollinosis.[47,69] In both trials, standardized Japanese cedar pollen extract was used at a monthly cumulative dose of 8,000 JAU, which contains approximately 10 μg of Cry j 1. This dosage is less than that reported in Europe, where a dose of 75,000 SQ-T (15 μg of a major grass allergen Phl p 5) was administered once daily for 18 weeks.[46] Unless the monthly cumulative dose is approximately $1/40^{th}$ of the amount required to be considered a major allergen (10/450 μg as a major allergen) in Japan, SLIT with an active treatment group against Japanese cedar pollinosis is still effective for improving quality of life and significantly ameliorates patients' SMS and symptom score during the pollen season. The up-regulation of the IL4-producing clone size specific to epitopes from Cry j 1 and Cry j 2[70] was reported to be significantly attenuated, and Cry j 1-specific IgG4 production was also significantly induced by active SLIT.[47] Furthermore, IL10-producing Tr1 cells were

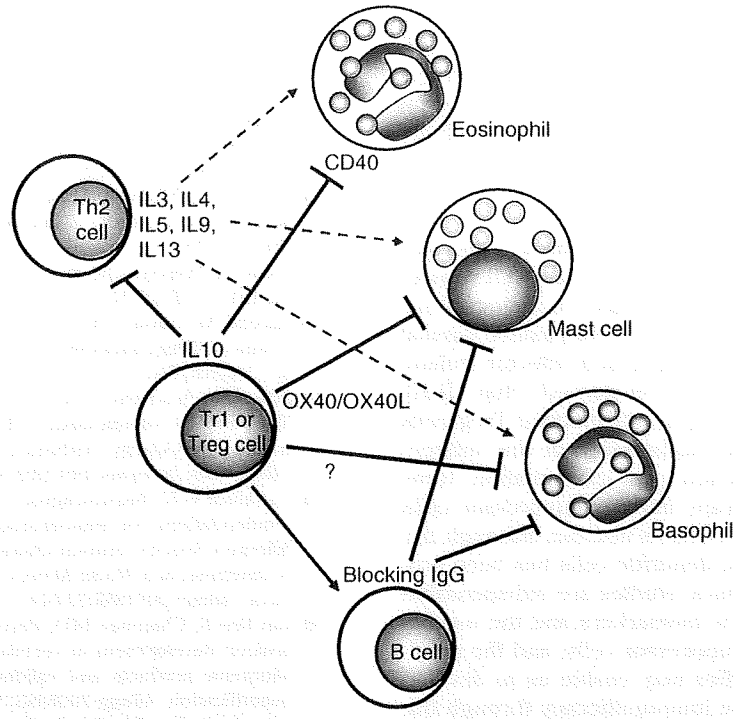

Fig. 3 Proposed roles of regulatory T cells on inflammatory cells in allergen-specific immunotherapy. Regulatory T cells, namely IL10-secreting Tr1 cells or adaptive/induced Treg cells, down-regulate inflammatory cells, directly or indirectly. Regulatory T cells down-regulate the activation of Th2 cells and subsequently Th2-type cytokine secretion. Regulatory T cells suppress the activation of inflammatory cells directly via their surface molecules and by secreting cytokines, and indirectly via the down-regulation of cytokine production in Th2 cells and by the activation of B cells to produce blocking IgG.

reported to be significantly increased in patients treated with SLIT compared with the levels in untreated patients and healthy subjects, and the proliferation of $CD4^+$ leukocytes stimulated with Cry j 1 and Cry j 2 was significantly suppressed by SLIT treatment in an IL10-dependent manner.[71] Supplementation with recombinant or native Cry j-allergens and/or updosing of the extract by bio-engineering may lead to more effective SLIT for treating pollinosis.

Another approach to safer immunotherapy is the use of oral immunotherapy using transgenic rice seed accumulating Cry j 1.[72] The generated transgenic rice plants expressed recombinant, structurally disrupted Cry j 1 peptides but spanned the entire Cry j 1 region as fusion proteins with the major rice storage protein glutenin. These fusion proteins aggregated with cysteine-rich prolamin and were deposited in endoplasmic reticulum-derived protein body I in rice seed. Transgenic rice expressing T cell epitopes from Cry j 1 and Cry j 2 successfully suppressed antigen-specific Th2-mediated IgE responses in a mouse model of allergic rhinitis.[73] Further clinical trials are needed to develop a rice-based edible vaccine as a tool for oral immunotherapy to control allergies.

An immunoregulatory liposome encapsulating the recombinant fusion protein of Cry j 1-Cry j 2 was manufactured as a novel vaccine for Japanese cedar pollinosis without risk of anaphylaxis.[74] The hybrid fusion allergen is expected to provide safer and more effective vaccines for immunotherapy. Vaccines using only T cell epitopes are also safer than native allergens, but there is wide variation among individual T cell epitopes. The fusion protein of major allergens covers all sequential T cell epitopes but is expected to have less IgE-binding capacity because its three-dimensional structure is disrupted in some B cell epitopes. Recombinant hybrid molecules using major allergens of timothy grass pollen induced stronger proliferation of PBMC in timothy-allergic patients than did mixtures of corresponding allergens, but still possess IgE-binding capacity and induce IgG production in sensitized mice.[75] In a mouse model sensitized with native Cry j 1 and Cry j 2, the vaccine that con-