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Original article

# Olopatadine hydrochloride in children: efficacy and safety for perennial allergic rhinitis

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**Key words:**
Antihistamine – Children – Double-blind study – Olopatadine – Perennial allergic rhinitis – Placebo

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## Abstract

**Objective:**
The efficacy of antihistamines in perennial allergic rhinitis in children has been evaluated in studies using active comparators, whereas placebo-controlled studies are very few. A randomized, multicenter, double-blind, parallel-group clinical study was carried out to evaluate the dose–response relationship and superiority of olopatadine hydrochloride over placebo in children aged 7 to 16 years with perennial allergic rhinitis.

**Methods:**
Subjects received twice daily treatment for two weeks with either olopatadine 2.5 mg, 5 mg or placebo after a one-week observation period. Efficacy was assessed based on the diary card score the subject (or guardian) recorded.

**Results:**
Of the 302 subjects randomized, two were excluded from analysis: one did not receive treatment; the other was not monitored for efficacy parameters. The remaining 300 subjects (97 in the placebo group, 103 in the olopatadine 2.5-mg group and 100 in the olopatadine 5-mg group) constituted the full analysis set (FAS) for the efficacy analysis. As a primary endpoint, the total three nasal symptom score (for sneezing, rhinorrhea and nasal congestion) at final assessment was compared with baseline or the score obtained in the observation period. The change from baseline was then tested using analysis of covariance (ANCOVA) with the baseline score as covariate. Williams' test was applied to the least squares means estimated from this ANCOVA model for each treatment group, resulting in showing the monotonicity Williams' test assumed. The total three nasal symptom score significantly improved in the 5-mg group compared with the placebo group ($p = 0.019$). In contrast, the 2.5-mg group did not differ statistically from the placebo group. Adverse events occurred in 33.7% (33/98 subjects) in the placebo group, 35.9% (37/103 subjects) in the 2.5-mg group and 35.0% (35/100 subjects) in the 5-mg group. There were no serious or severe adverse events.

**Conclusions:**
Olopatadine hydrochloride 5 mg twice daily is an effective and safe treatment for perennial allergic rhinitis in children.

## Introduction

Allergic rhinitis, the prevalence of which is increasing worldwide, is known to affect school performance and work productivity and sometimes to impair sleep[1]. It is a common pediatric disease that induces or aggravates asthma, sinusitis, otitis media and other diseases[2]. Treating children with allergic rhinitis is all the more difficult because they may not accurately describe their symptoms[2,3].

Most cases of allergic rhinitis in Japanese children are perennial. Diagnosis and medical treatment of pediatric allergic rhinitis are made according to the guideline provided for adults. Histamine plays an important role in producing nasal symptoms including sneezing and rhinorrhea, and second-generation

antihistamines are commonly used for the relief of these symptoms. The need for antihistamines is growing in children as well as in adults.

Olopatadine hydrochloride, synthesized by Kyowa Hakko Kogyo Co., Ltd (currently Kyowa Hakko Kirin Co., Ltd), is an antiallergic that acts primarily against the histamine $H_1$ receptor, inhibits release of mediators such as thromboxanes and leukotrienes and exhibits inhibitory effect on the release of tachykinins known to contribute to exacerbation of allergic inflammation[4]. Its oral preparation has been approved for adult allergic rhinitis, urticaria and pruritic skin diseases first in Japan, while ophthalmic and nasal preparations have been developed mainly in the USA. The efficacy and safety of olopatadine are now acknowledged worldwide[5–9].

With an aim to obtain approval for pediatric use of olopatadine hydrochloride in Japan, a randomized, multicenter, double-blind, parallel-group clinical study was carried out to evaluate the superiority over placebo and safety of oral olopatadine in children aged 7 to 16 years with perennial allergic rhinitis. Subjects received twice daily treatment for two weeks with either olopatadine 2.5 mg, 5 mg or placebo. Assessment was based on the diary card score the subject (or guardian) recorded concerning his/her nasal allergy symptoms.

The study was conducted at 31 sites in Japan between July 2005 and March 2006 in accordance with the principles described in the Declaration of Helsinki, Good Clinical Practice (GCP) and the protocol that had been approved by each institutional review board.

# Methods

## Study design

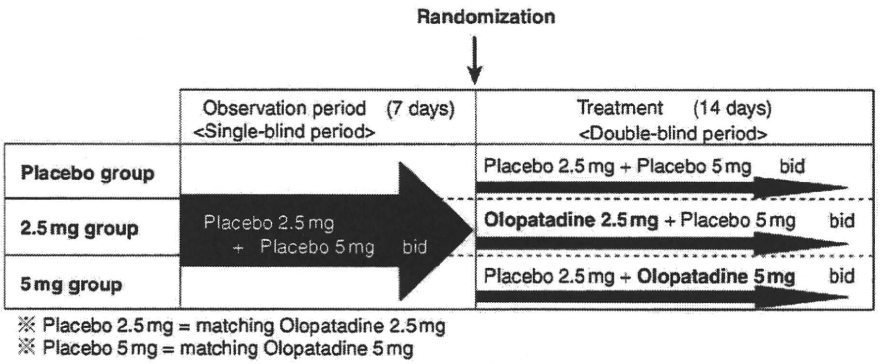
The study period was three weeks, consisting of a one-week observation period (single-blind period) and a two-week double-blind period as shown in Table 1.

## Subjects and methods

Children aged 7 to 16 years with perennial allergic rhinitis were recruited if they were allergic to house dust or mites on the skin test (intradermal or scratch test) or test for serum specific IgE antibodies and if they showed a positive nasal challenge test or a positive eosinophil count in nasal discharge in accordance with the criteria shown in Tables 2 and 3. Children who had concurrent severe bronchial asthma or atopic dermatitis or in whom requirement for antihistamines, leukotriene antagonists, steroids or any other drug that acted on nasal symptoms was anticipated were excluded from the study. When the observation and the double-blind period were in the pollen season (pollen from cocksfoot, timothy grass, ragweed, artemisia, cedar, cypress, Alnus japonica and white birch) children who were positive to any of the pollen antigens were also excluded from the study. All subjects received placebo in a single-blind fashion in the observation period to identify subjects who showed nasal symptoms suitable for evaluation and who could keep a diary as instructed. Subjects were randomized to double-blind treatment if they had a mean rhinorrhea score of 2 or more and a mean total three nasal symptom score of 4 or more for the last four days prior to randomization as determined using Table 2 and unless their diary card data were missing for two days or more.

The following medications were prohibited after laboratory tests prior to the observation period through the end of the double-blind period (or until discontinuation): chemical mediator release inhibitors, antihistamines, thromboxane $A_2$ inhibitors, thromboxane $A_2$ antagonists, leukotriene antagonists, Th2 cytokine inhibitors, corticosteroids, α-sympathetic stimulants, anti-cholinergics, drugs for nonspecific modulation therapy, biological preparations, glycyrrhizine products, herbal medications, drugs for bronchial asthma (inhaled corticosteroids), vasodilators ($\beta_2$-stimulants, theophylline products), expectorants (stimulants of airway secretion, airway mucolytics, airway

Table 1. Study design.



mucus adjusters), centrally acting non-narcotic antitussives (approved for use in bronchial asthma), antitussive–expectorant combinations (approved for use in bronchial asthma) and immunosuppressants for oral, injection, nasal, inhalation, external or suppository use. The use of external or ocular corticosteroids was also prohibited.

Voluntary written informed consent to participate in the study was obtained from all guardians. All subjects provided voluntary assent to study participation.

After written consent was obtained from guardians, baseline characteristics and eligibility of subjects were recorded and hematology, blood chemistry and urinalysis performed. On the first day of the observation period, eligibility was assessed on the basis of diagnosis, body weight and laboratory findings. Once eligibility was confirmed, subjects/guardians were supplied with nasal allergy diary cards and the study drug to be taken during the observation period. They were fully instructed how to record and manage the diary. On the first day of the double-blind period, diary records and compliance with study medication during the observation period were assessed; subjects/guardians were asked whether the subject had experienced cold symptoms during the observation period. Subjects who fulfilled all of the inclusion/exclusion criteria were considered eligible and supplied with a double-blind medication designated as drug number. Rhinoscopic findings and diary records were checked on the first day of the double-blind period and at the week-1 and week-2 visits. Diary cards were collected and hematology, blood chemistry and urinalysis performed at the week-2 visit.

## Criteria for evaluation

### Efficacy

The severity of nasal symptoms (sneezing, rhinorrhea, nasal congestion and disturbance of daily life) was scored as shown in Table 4 and recorded on diary cards. Mean total three symptom score and mean individual symptom scores were calculated using diary cards on which all of the three main nasal symptoms were rated. Diary scores on the last four days prior to each assessment time point were used to calculate daily mean score. Mean scores obtained in the observation period were considered as baseline and the change from baseline over the double-blind period was calculated at each assessment.

The primary analysis was carried out at final assessment at the end of the two-week treatment (or at the end of the one-week treatment if data at the end of the two-week treatment were missing).

The primary efficacy endpoint was the change from baseline in total three nasal symptom score (for sneezing, rhinorrhea and nasal congestion) and secondary endpoints included changes in individual symptom scores (sneezing, rhinorrhea, nasal congestion and disturbance of daily life), individual nasal local finding scores and severity score. Nasal local findings at each assessment time point were scored according to the criteria given in Table 5. Mean individual symptom scores (for sneezing, rhinorrhea and nasal congestion) in the observation, week-1 treatment and week-2 treatment periods calculated from diary records were used to classify and score the severity of allergic rhinitis as shown in Table 6.

### Safety

Safety was assessed on the basis of adverse events and reactions newly developing or aggravated in the double-blind period. The sponsor coded reported adverse events using the ICH Medical Dictionary for Regulatory Activities/Japanese Edition (MedDRA/J version 9.0). Laboratory data were assessed for abnormalities suggestive of adverse event and for changes over time.

Table 3. Evaluation criteria of the test for serum specific IgE antibodies.

| Test \ Evaluation criteria | Positive | False positive/negative |
|---|---|---|
| RAST, CAP-RAST, LUMIWARD, SIST, AlaSTAT | Class 2 or higher | Class 1 or Class 0 |
| MAST | Class 1 or higher | Class 0 |

Table 2. Diagnostic criteria for allergic rhinitis.

| Test \ Severity | +++ | ++ | + | ± | − |
|---|---|---|---|---|---|
| | Positive | | | Negative | |
| Intradermal test* | Erythema: ≥ 41 mm Wheal: ≥16 mm | Erythema: 40–20 mm Wheal: 15–10 mm | Erythema: 40–20 mm Wheal: ≤9 mm | | Erythema: ≤19 mm Wheal: ≤9 mm |
| Nasal challenge test** | Three symptoms, particularly more than 6 sneezes | Three symptoms | Two symptoms | One symptom | 0 |
| Eosinophil count in nasal discharge | Present in groups | Between (+++) and (+) | Found by weak magnification | | 0 |

*A scratch (prick) test is considered positive when a wheal or erythema is more than twice that of control in diameter or an erythema is greater than 10 mm or a wheal greater than 5 mm in diameter after 15 to 30 minutes.
**Three symptoms: (1) Sneezing, nasal itching, (2) Swelling and pallor of the lower nasal turbinate membrane, (3) Watery secretion.

Table 4. Severity of nasal symptoms[10].

| Symptom \ Severity | ++++ (Score: 4) | +++ (Score: 3) | ++ (Score: 2) | + (Score: 1) | − (Score: 0) |
|---|---|---|---|---|---|
| Sneezing (Mean episodes/day) | 21 or more | 20 to 11 | 10 to 6 | 5 to 1 | 0 |
| Rhinorrhea (Mean episodes/day) | 21 or more | 20 to 11 | 10 to 6 | 5 to 1 | 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 |
| Disturbance of daily life | Unable to eat, play, study or sleep | Eating, playing or studying difficult | Eating, playing or studying a little difficult | Eating, playing or studying not difficult but efforts required | No difficulty with eating, playing or studying |

## Statistical analysis

For the efficacy analysis, the full analysis set (FAS) was defined as all randomized subjects who received at least one dose of study medication in the double-blind period and who were monitored for efficacy parameters at least once in each of the observation and double-blind periods. The safety population consisted of all randomized subjects who received at least one dose of study medication in the double-blind period and from whom post-dose safety data were available.

It was anticipated that baseline scores obtained in the observation period would influence the primary and secondary endpoints, which were assessed based on the mean change in symptom scores from baseline to each assessment time point. The primary analysis was therefore performed using an analysis of covariance (ANCOVA) with the baseline score as covariate. For the primary endpoint, alternative hypotheses 'mean for the placebo group $\leq$ mean for the olopatadine 2.5-mg group $\leq$ mean for the olopatadine 5-mg group (wherein at least one $\leq$ was $<$)' and 'mean for the placebo group $<$ mean for the olopatadine 2.5-mg group' were tested using Williams' test at a one-sided significance level of 2.5% to assess dose–response relationship.

The level of significance was 5% for two-sided exploratory analysis of efficacy and safety. Whether there was demographic bias or interaction was tested with a two-sided significance level of 15%.

# Results

## Demographics

Of the 413 subjects who were screened in the observation period, 111 did not meet the inclusion/exclusion criteria and were withdrawn from the study, while 302 were randomized to treatment. Failure to meet the criterion: 'a mean rhinorrhea score of 2 or more and a mean total three nasal symptom score of 4 or more for the last four days prior to randomization' was the most common reason for withdrawal (96 subjects). Of the randomized subjects, 93 in the placebo group, 98 in the 2.5-mg group and 93 in the 5-mg group completed the study, while six in the placebo group, five in the 2.5-mg group and seven in the 5-mg group discontinued treatment (Table 7).

Two subjects randomized to placebo were excluded from efficacy analysis because one did not receive double-blind treatment and the other was not monitored for efficacy parameters. The FAS for the efficacy analysis included 300 subjects (97 on placebo, 103 on 2.5 mg and 100 on 5 mg). The one subject randomized but not treated was also excluded from safety evaluation and the safety

Table 5. Severity of local findings.

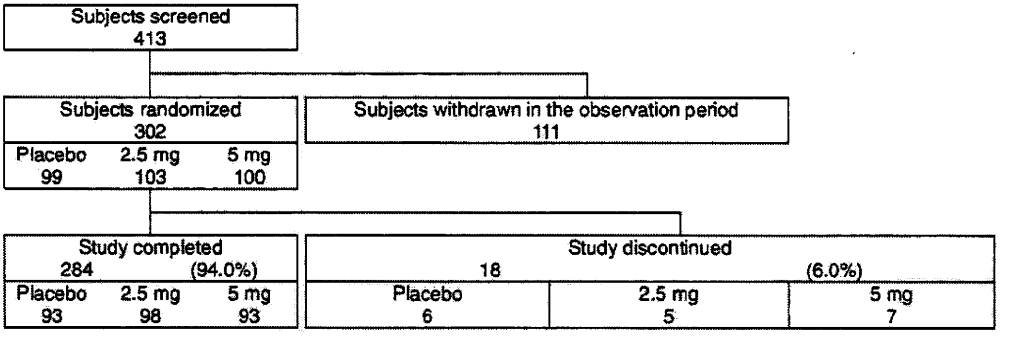
| Findings \ Severity | +++ (Score: 3) | ++ (Score: 2) | + (Score: 1) | − (Score: 0) |
|---|---|---|---|---|
| Swelling of the inferior turbinate mucosa | Middle turbinate not visible | Intermediate between +++ and + | Visible up to the center of the middle turbinate | No swelling |
| Watery secretion | Filling the nasal meatus | Intermediate between +++ and + | Seen adhering to the mucosa in small quantities | None |

Table 6. Severity.

| Severity | | Sneezing or rhinorrhea (whichever is more severe) | | | | |
|---|---|---|---|---|---|---|
| | | ++++ | +++ | ++ | + | − |
| Nasal congestion | ++++ | Most severe | Most severe | Most severe | Most severe | Most severe |
| | +++ | Most severe | Severe | Severe | Severe | Severe |
| | ++ | Most severe | Severe | Moderate | Moderate | Moderate |
| | + | Most severe | Severe | Moderate | Mild | Mild |
| | − | Most severe | Severe | Moderate | Mild | No symptom |

Score: Most severe, 4; Severe, 3; Moderate, 2; Mild, 1; No symptom, 0.

Table 7. Subject participation.




population consisted of 301 subjects (98 on placebo, 103 on 2.5 mg and 100 on 5 mg).

Demographic and other baseline characteristics of the FAS are summarized in Table 8. A two-sided test at the 15% significance level revealed no bias in the distribution of sex, age and body weight among three groups.

## Efficacy

Primary efficacy endpoint (changes from baseline in total three nasal symptom scores – sneezing, rhinorrhea and nasal congestion) were compared using ANCOVA with the baseline score as covariate. The least squares mean estimated for each treatment group from this ANCOVA model was evaluated using Williams' test. The results are shown in Tables 9 and 10.

The least squares mean for the changes from baseline to final assessment in total three symptom score was −0.88, −0.95 and −1.38 for the placebo, 2.5-mg and 5-mg groups, respectively, showing the monotonicity of dose response Williams test assumed. The difference in least squares mean between the 5-mg and placebo groups (placebo group – 5-mg group) was 0.51 (95% confidence interval: 0.04 to 0.98) with a $p$ value of 0.019 by Williams' test, demonstrating that olopatadine 5 mg significantly reduced total three symptom score compared with placebo. In contrast, there was no significant difference between the 2.5-mg and placebo groups since the difference in least squares mean between the groups (placebo group - 2.5-mg group) was 0.08 (95% confidence interval: −0.39 to 0.54) with a $p$ value of 0.375.

The ANCOVA with the total three symptoms score at baseline as covariate yielded least squares

Table 8. Subject characteristics.

| | Treatment | Placebo | 2.5 mg | 5 mg | p |
|---|---|---|---|---|---|
| | Number of subjects | 97 | 103 | 100 | |
| Sex | Male | 65 (67.0%) | 67 (65.0%) | 63 (63.0%) | [a]0.835 |
| | Female | 32 (33.0%) | 36 (35.0%) | 37 (37.0%) | |
| Age (years) | Mean ± SD | 10.6 ± 2.5 | 10.9 ± 2.7 | 10.9 ± 2.8 | [b]0.707 |
| | Median | 11.0 | 10.0 | 11.0 | [c]0.827 |
| | Min–max | 7–16 | 7–16 | 7–16 | |
| Body weight (kg) | Mean ± SD | 38.27 ± 12.10 | 38.57 ± 13.95 | 39.43 ± 13.45 | [b]0.814 |
| | Median | 37.50 | 35.50 | 36.30 | [c]0.835 |
| | Min–max | 20.0–79.0 | 20.0–96.0 | 21.0–74.4 | |

[a]Fisher's exact test.
[b]One-way analysis of variance.
[c]Kruskal–Wallis test.
SD: Standard deviation.

Table 9. Analysis of covariance for changes from baseline to final assessment in total three nasal symptom score (sneezing, rhinorrhea and nasal congestion).

| | | | Placebo ($n = 97$) | 2.5 mg ($n = 103$) | 5 mg ($n = 100$) |
|---|---|---|---|---|---|
| Observation period (baseline) | Descriptive statistics | Number of subjects | 97 | 103 | 100 |
| | | Mean ± SD | 5.99 ± 1.17 | 6.09 ± 1.20 | 6.14 ± 1.44 |
| | | Median | 6.00 | 6.00 | 6.00 |
| | | Min ~ max | 4.0 ~ 10.8 | 4.0 ~ 9.5 | 4.0 ~ 10.8 |
| Final assessment score – baseline score | Descriptive statistics | Number of subjects | 97 | 103 | 100 |
| | | Mean ± SD | −0.84 ± 1.58 | −0.96 ± 1.70 | −1.41 ± 1.99 |
| | | Median | −0.80 | −0.70 | −1.50 |
| | | Min ~ max | −5.5 ~ 3.0 | −5.5 ~ 4.3 | −5.0 ~ 6.7 |
| | ANCOVA | Least squares mean | −0.88 | −0.95 | −1.38 |
| | | 95% CI | [−1.21, −0.54] | [−1.27, −0.63] | [−1.71, −1.05] |
| | | $p^a$ | <0.001* | <0.001* | <0.001* |
| | ANCOVA (placebo -olopatadine) | Least squares mean | – | 0.08 | 0.51 |
| | | 95% CI | – | [−0.39, 0.54] | [0.04, 0.98] |
| | | $p^b$ | – | 0.750 | 0.034* |

[a]$p$ value by the two-sided $t$-test evaluating the null hypothesis that final assessment-baseline = 0.
[b]$p$ value by the two-sided $t$-test evaluating the null hypothesis that the score for the placebo group the-olopatadine 2.5-mg (5-mg) group = 0.
*$p < 0.05$.

Table 10. Williams' test for changes from baseline to final assessment in total three nasal symptom score (sneezing, rhinorrhea and nasal congestion).

| Final assessment-baseline | | Placebo $n = 97$ | 2.5 mg $n = 103$ | 5 mg $n = 100$ |
|---|---|---|---|---|
| Least squares mean[a] | | −0.88 | −0.95 | −1.38 |
| Difference from the placebo group | Least squares mean | | 0.08 | 0.51 |
| | 95% CI | | [−0.39, 0.54] | [0.04, 0.98] |
| | $p^b$ | | 0.375 | 0.019* |

[a]Estimated from a model with treatment arm (placebo/2.5 mg/5 mg) as factor and the total three symptom score at baseline as covariate.
[b]$p$ value by Williams' test (one-sided).
5-mg group: $p$ value by the one-sided test evaluating the alternative hypothesis that mean for the placebo group ≤ mean for the olopatadine 2.5-mg group ≤ mean for the olopatadine 5-mg group (at least one ≤ is <).
2.5-mg group: $p$ value by the one-sided test evaluating the alternative hypothesis that mean for the placebo group < mean for the olopatadine 2.5-mg group.
*$p < 0.025$.

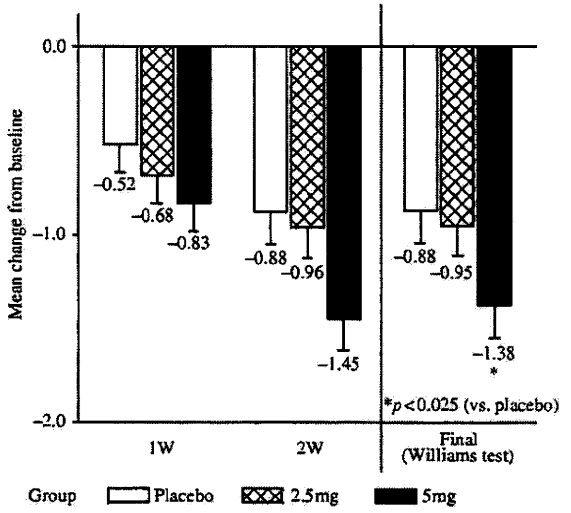



Figure 1. Transition of mean change from baseline in total nasal symptom scores.

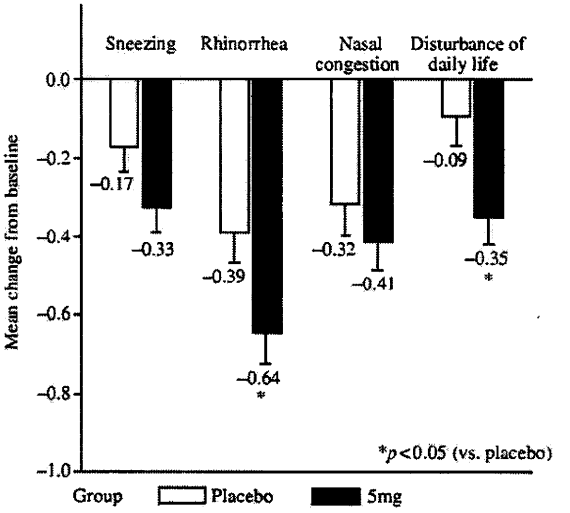



Figure 2. Mean change from baseline in individual nasal symptom scores at final assessment.

means ± standard error, which are presented in Figure 1 by assessment time point. The total score was lower after the two-week treatment than after the one-week treatment in all three groups.

## Secondary efficacy endpoints

Least squares means for the changes from baseline to final assessment in individual symptom scores (for sneezing, rhinorrhea, nasal congestion and disturbance of daily life) indicated that the scores for sneezing, rhinorrhea and nasal congestion at final assessment were reduced compared with those at baseline in all three groups. The difference in least squares mean at final assessment between the 5-mg and placebo groups was 0.16 (95% confidence interval: −0.02 to 0.33) for sneezing, 0.25 (95% confidence interval: 0.04 to 0.47) for rhinorrhea, 0.09 (95% confidence interval: −0.12 to 0.31) for nasal congestion and 0.26 (95% confidence interval: 0.05 to 0.46) for disturbance of daily life. Olopatadine 5 mg significantly improved rhinorrhea and disturbance of daily life at final assessment compared with placebo ($p = 0.022$ for rhinorrhea; $p = 0.013$ for disturbance of daily life) (Figure 2).

The ANCOVA for the changes from baseline in individual scores for local findings (swelling and color of the inferior turbinate mucosa, watery secretion and appearance of nasal discharge) performed in the same fashion as for the primary endpoint showed that the scores at final assessment were reduced compared with those at baseline for all findings and in all groups. No differences were observed among the three groups.

The ANCOVA for the changes from baseline in severity scores showed that olopatadine 5 mg improved allergic rhinitis compared with placebo ($p = 0.006$).

## Safety

Adverse events occurred at similar rates across three groups: 33.7% (33/98 subjects) in the placebo group, 35.9% (37/103) in the olopatadine 2.5-mg group and 35.0% (35/100) in the olopatadine 5-mg group (Table 11). There were no serious or severe adverse events. The most frequent adverse event was nasopharyngitis, which occurred in 8.2% (8/98) on placebo, 6.8% (7/103) on 2.5 mg and 6.0% (6/100) on 5 mg, followed by alanine aminotransferase increasing, which occurred in 3.1% (3/98) on placebo, 3.9% (4/103) on 2.5 mg and 7.0% (7/100) on 5 mg. White blood cell count increased in 2.9% (3/103) on 2.5 mg and 4.0% (4/100) on 5 mg. The incidence of the adverse event somnolence was 1.0% (1/98) in the placebo group, 2.9% (3/103) in the 2.5-mg group and 1.0% (1/100) in the 5-mg group. Somnolence was mild in severity in all of these subjects.

## Discussion

Olopatadine hydrochloride alleviates allergic reactions through its histamine $H_1$ receptor antagonist activity. Its oral preparation has been approved for adult allergic rhinitis, urticaria and pruritic skin diseases first in Japan. Since then ophthalmic and nasal preparations have been developed mainly in the USA and are now used worldwide. Oral olopatadine was demonstrated to be a histamine $H_1$ receptor antagonist more potent than other drugs in the same therapeutic class[11] and to be effective in the treatment of the most common seasonal allergic rhinitis in Japan, Japanese cedar pollinosis[12].

This is a placebo-controlled study to evaluate the efficacy and safety of olopatadine in children with perennial

Table 11. Frequency of adverse event occurrence (occurring in two or more subjects).

| AEs (Preferred Term) | Placebo ($n=98$) | | 2.5 mg ($n=103$) | | 5 mg ($n=100$) | |
|---|---|---|---|---|---|---|
| Total | Number | % | Number | % | Number | % |
| | 33 | 33.7 | 37 | 35.9 | 35 | 35.0 |
| Abdominal pain | 2 | 2.0 | 2 | 1.9 | 0 | 0.0 |
| Diarrhea | 2 | 2.0 | 3 | 2.9 | 2 | 2.0 |
| Acute tonsillitis | 1 | 1.0 | 2 | 1.9 | 0 | 0.0 |
| Gastroenteritis | 3 | 3.1 | 1 | 1.0 | 1 | 1.0 |
| Influenza | 3 | 3.1 | 1 | 1.0 | 1 | 1.0 |
| Nasopharyngitis | 8 | 8.2 | 7 | 6.8 | 6 | 6.0 |
| Pharyngitis | 1 | 1.0 | 1 | 1.0 | 3 | 3.0 |
| Rhinitis | 0 | 0.0 | 0 | 0.0 | 2 | 2.0 |
| Laryngopharyngitis | 0 | 0.0 | 3 | 2.9 | 1 | 1.0 |
| Alanine aminotransferase increased | 3 | 3.1 | 4 | 3.9 | 7 | 7.0 |
| Aspartate aminotransferase increased | 3 | 3.1 | 2 | 1.9 | 1 | 1.0 |
| Blood urea increased | 2 | 2.0 | 0 | 0.0 | 1 | 1.0 |
| Blood urine present | 0 | 0.0 | 2 | 1.9 | 0 | 0.0 |
| White blood cell count increased | 0 | 0.0 | 3 | 2.9 | 4 | 4.0 |
| Protein urine present | 2 | 2.0 | 0 | 0.0 | 1 | 1.0 |
| Headache | 2 | 2.0 | 3 | 2.9 | 1 | 1.0 |
| Somnolence | 1 | 1.0 | 3 | 2.9 | 1 | 1.0 |
| Cough | 0 | 0.0 | 2 | 1.9 | 1 | 1.0 |
| Upper respiratory tract inflammation | 0 | 0.0 | 2 | 1.9 | 2 | 2.0 |

allergic rhinitis. Approximately 70 to 80% of Japanese children are likely to develop mite allergy and most clinical studies in children aim at the more prevalent category, perennial allergic rhinitis[13,14]. In conducting the study, the authors tried to eliminate seasonal factors that might affect study results by excluding patients who showed a positive reaction to the pollens that were thought to be dispersing during the study period. The severity of allergic rhinitis was determined on the basis of the three main symptoms, sneezing, rhinorrhea and nasal congestion. They are commonly used as a comprehensive indicator of the severity of allergic rhinitis in clinical settings in Japan and especially helpful in therapeutic decision making for children. In this study, they served as one of the criteria on which to assess subject eligibility and efficacy. Subjects or guardians were instructed to record the number of sneezes, frequency of nose blow and degree of nasal congestion on diary cards each day, which were scored to represent the severity of nasal symptoms. Evident nasal symptoms and accurate diary data were considered requirements for the subject to progress from the seven-day observation period to the double-blind phase. Subjects were therefore randomized to double-blind treatment if they had a mean rhinorrhea score of 2 or more and a mean total three symptom score of 4 or more for the last four days prior to the double-blind phase, unless their diary card data were missing for two days or more.

Oral olopatadine 5 mg administered for 14 days was superior to placebo in reducing total three nasal symptom score. The International Conference on Harmonization Notes for Guidance on Good Clinical Practice (E4: Dose–Response Information to Support Drug Registration) providing that including a placebo group is desirable in dose–response studies is applied to this study. Though some authors have reported placebo-controlled studies in children with seasonal allergic rhinitis[15–17], few placebo-controlled studies have been published concerning pediatric perennial allergic rhinitis. Levocetirizine significantly improved Total 4 Symptoms Score (the sum of scores for sneezing, rhinorrhea, nasal pruritus and ocular pruritus), 50% response rate and other efficacy variables compared with placebo in a study with approximately 150 subjects per group[18]. Neither placebo-controlled studies of oral olopatadine nor studies similar to this study of other drugs in the same therapeutic class have been reported. This study seems to be of particular interest as it is the only study that shows the superiority of olopatadine over placebo in three main nasal symptoms in children and as it demonstrates the histamine $H_1$ receptor antagonist activity of olopatadine.

No serious adverse events occurred during the study. There were no large differences in the incidence of adverse events between olopatadine (2.5 and 5 mg) treated and placebo-treated groups. The most frequent adverse event was nasopharyngitis and its high incidence was possibly attributable to the facts that the subjects were children and that they participated in the study in winter. Somnolence, an adverse event commonly reported with histamine $H_1$ receptor antagonists, was seen only in a limited number of subjects in this study; the incidence was similar between olopatadine and placebo groups and the severity was mild.

These results demonstrate that olopatadine hydrochloride 5 mg twice daily is an effective and safe treatment for perennial allergic rhinitis in children.

## Conclusion

A randomized, multicenter, double-blind, parallel-group clinical study was carried out to evaluate the dose–response relationship and superiority of olopatadine hydrochloride over placebo in children aged 7 to 16 years with perennial allergic rhinitis. As a primary endpoint, the total three nasal symptom score (for sneezing, rhinorrhea and nasal congestion) at final assessment was compared with baseline or the score obtained in the observation period. The change from baseline was then tested using analysis of covariance (ANCOVA) with the baseline score as covariate. Williams' test was applied to the least squares means estimated from this ANCOVA model for each treatment group, resulting in showing the monotonicity Williams' test assumed. The total three nasal symptom score significantly improved in the 5-mg group compared with the placebo group ($p = 0.019$). Adverse events occurred in 33.7% (33/98 subjects) in the placebo group, 35.9% (37/103 subjects) in the 2.5-mg group and 35.0% (35/100 subjects) in the 5-mg group. There were no serious or severe adverse events. These results demonstrate that olopatadine hydrochloride 5 mg twice daily is an effective and safe treatment for perennial allergic rhinitis in children.

## Transparency

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**Declaration of financial/other relationships**

K.O. has disclosed that he acted as a consultant and medical advisor for this study. M.O. has disclosed that he acted as a consultant for this study. H.M. and K.K. have disclosed that they are employees of Kyowa Hakko Kirin Co., Ltd.

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## References

1. Bousquet J. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 Update (in collaboration with the World Health Organization, GA$^2$LEN and AllerGen). Allergy 2008;63:30-43
2. Fireman P. Therapeutic approaches to allergic rhinitis: treating the child. J Allergy Clin Immunol 2000;105:S616-21
3. Lack G. Pediatric allergic rhinitis and comorbid disorders. J Allergy Clin Immunol 2001;108:S9-15
4. Kaise T, Karasawa A. Olopatadine hydrochloride (Allelock®): inhibitory effect on sensory nerve activation and effectiveness in reducing itching associated with allergic rhinitis, urticaria and skin disease. Allergy Pract 2002;22:50-63
5. Shah SR, Nayak A, Ratner P, et al. Effect of olopatadine hydrochloride nasal spray 0.6% in the treatment of seasonal allergic rhinitis: a phase III, multicenter, randomized, double-blind, active- and placebo-controlled study in adolescent and adults. Clin Ther 2009;31:99-107
6. Ratner PH, Hampel FC, Amar NJ, et al. Safety and efficacy of olopatadine hydrochloride nasal spray for the treatment of seasonal allergic rhinitis to mountain cedar. Ann Allergy Asthma Immunol 2005;95:474-9
7. Meltzer EO, Hampel FC, Ratner PH, et al. Safety and efficacy of olopatadine hydrochloride nasal spray for the treatment of seasonal allergic rhinitis. Ann Allergy Asthma Immunol 2005;95:600-6
8. Berger W, Abelson MB, Gomes PJ, et al. Effects of adjuvant therapy with 0.1% olopatadine hydrochloride ophthalmic solution on quality of life in patients with allergic rhinitis using systemic or nasal therapy. Ann Allergy Asthma Immunol 2005;95:361-71
9. Abelson MB, Turner D. A randomized, double-blind, parallel-group comparison of olopatadine 0.1% ophthalmic solution versus placebo for controlling the signs and symptoms of seasonal allergic conjunctivitis and rhinoconjunctivitis. Clin Ther 2003;25:931-47
10. Kimihiro Okubo, Kohtaro Baba. A double-blind non-inferiority clinical study of montelukast, a cysteinil leukotriene receptor 1 antagonist, compared with pranlukast in patients with seasonal allergic rhinitis. Allergol Int 2008; 57:383-90
11. Hitoshi Takahashi, Yan Zhang, Eishin Morita. Evaluation of the antihistamine effects of olopatadine, cetirizine and fexofenadine during a 24 h period: a double-blind, randomized, crossover, placebo-controlled comparison in skin responses induced by histamine iontophoresis. Arch Dermatol Res 2008; 300:291-5
12. Enomoto T, Lu H-Q, Yin M, et al. Evaluation of the efficacy and safety of olopatadine and fexofenadine compared with placebo in Japanese cedar pollinosis using an environmental exposure unit. J Investig Allergol Clin Immunol 2009;19:299-305
13. Ishikawa T, Tanigawara Y, Suzuki I, et al. A phase-III double-blind comparative study of loratadine syrup in children with perennial allergic rhinitis – non-inferiority versus ketotifen fumarate dry syrup. J Clin Therap Med 2007; 23:965-90
14. Baba K. Efficacy and safety of fexofenadine hydrochloride in children with perennial allergic rhinitis – comparison with ketotifen fumarate dry syrup. Practica Oto-Rhino-Laryngologica 2007;Suppl. 119:1-20
15. Wahn U, Meltzer EO, Finn Jr AF, et al. Fexofenadine is efficacious and safe in children (aged 6–11 years) with seasonal allergic rhinitis. J Allergy Clin Immunol 2003;111:763-9
16. de Blic J, Wahn U, Billard E, et al. Levocetirizine in children: evidenced efficacy and safety in a 6-week randomized seasonal allergic rhinitis trial. Pediatr Allergy Immunol 2005;16:267-75
17. Pearlman DS, Lumry WR, Winder JA, et al. Once-daily cetirizine effective in the treatment of seasonal allergic rhinitis in children aged 6 to 11 years: a randomized, double-blind, placebo-controlled study. Clin Pediatr 1997; 36:209-15
18. Potter PC, for the Paediatric Levocetirizine Study Group. Efficacy and safety of levocetirizine on symptoms and health-related quality of life of children with perennial allergic rhinitis: a double-blind, placebo-controlled randomized clinical trial. Ann Allergy Asthma Immunol 2005;95:175-80

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分担研究報告書（総合）

# 自動花粉測定器の精度改善に向けての検討と主要花粉抗原ペプチドを用いた特異的Ｔ細胞クローンの測定についての検討


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|---|---|---|---|
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研究要旨

花粉曝露の回避は花粉症治療の第一歩であり、精度の高い花粉飛散情報の有用性は高い。リアルタイムで花粉測定が可能な自動花粉測定器の活用には期待されてはいるものの、従来の検討から自動花粉測定器には空中浮遊粒子との識別に問題があること、機種によっては感度に大きな問題あることが明らかで、実際の運用には改善が必須と考えられている。そこで、大気中の全粒子の測定値と、その中で花粉と識別した粒子の両方の表示が可能な自動花粉測定器を用いて、自動花粉測定器の課題である空中非花粉粒子との識別能の向上について検討を進め、花粉見逃し率、花粉誤認率を算定して、補正を加えることで自動花粉測定器の精度向上を図る検討を実施した。その結果、自動花粉測定器による測定値に補正式を加えることで、相関係数の向上が確認され、特に花粉飛散が少なく、従来花粉自動測定器で誤差が大きくみられていた時期の測定値に大きな改善がみられた。一方、抗原特異的なメモリーT 細胞クローンの測定方法について検討を進めたが、スギ花粉合成ペプチドを用いたスギ花粉特異的Ｔ細胞クローンの測定は、自己樹状細胞を抗原でパルスして用いる従来の検出法と比較して簡便であり、またダニ抗原合成ペプチドを用いた検討でも、ダニ特異的Ｔ細胞クローンの測定が簡便に出来、臨床応用への展開が期待される。


## A.　研究目的

正確な花粉飛散情報の提供は患者のセルフケアに有用であるが、リアルタイムで花粉測定が可能な自動花粉測定器の活用には期待が集まって、実際に様々な機種が使用されている。しかし、感度、特異性といった基本的な性能について十分な検討、評価がなされていないまま使用されている。機種によっては明らかに降雪の影響を受けていたり、非常に感度が低いものもみられる。また、これまでの検討で空中浮遊微粒子には、径が30μ、球形に近く表面が比較的滑らかで花粉の形状に似ているものが少なくないことから、現状の自動花粉測定器の花粉と微粒子との鑑別にどうしても限界がみられてしまう。そこで、大気中の全粒子の測定値と、その中で花粉と識別した粒子の両方の表示が可能な自動花粉測定器を用いて、自動花粉測定器の課題である空中非花粉粒子との識別能の向上について検討を進めてきた。すなわち、花粉見逃し率、花粉誤認率を算定して補正を加えることで自動花粉測定器の精度向上が図った。また、花粉症の発症や治療効果の客観的な評価が可能なバイオマーカーの検出が求められているが、花粉抗原特異的なメモリーT 細胞クローンの測定方法について抗原ペプチドを用いる簡便な方法の確立について検討を行った。

## B.　研究方法

測定全粒子数と花粉測定数の両値の表示が可能で、かつ比較的安価な自動花粉測定器（神栄）を用いて、スギ花粉飛散開始直前でまだ花粉飛散がほとんど見られない1月に千葉市及び成田市での測定データを検討した。この花粉非飛散期のデータから空中浮遊粒子を花粉と識別してしまう花粉誤認率を算出し、機種毎の花粉見逃し率を利用して新たな補正マトリックスを作成した。この補正式を利用してスギ花粉飛散期の自動花粉測定器による検出花粉数とダーラム式による花粉測定結果について相関を検討した。

一方、スギ花粉の主要抗原であるCry j1から4種類、Cry j2から3種類のペプチドを選定してリジンでそれぞれのペプチドを連結させた合成ペプチドを利用して、またダニ主要抗原のDer f 1,2からそれぞれ7種類のペプチドを作成、連結させて、直接患者リンパ球に反応させてスギ花粉症患者、ダニ通年性アレルギー性鼻炎患者末梢血中の抗原特異的Ｔ細胞のクローンサイズの測定をELISPOT 法により行い、細胞の特異性、それぞれの患者自身の樹状細胞をスギ、あるいはダニ粗抗

原で処理してリンパ球と反応させた場合との違い、クローンサイズの季節変動などを検討した。
（倫理面への配慮）
アレルギー性鼻炎患者からの採血については、目的を十分に説明して同意を文書により得て実施した。また、試験計画は千葉大学の倫理員会に申請して許可を得た後に実施した。

C. 研究結果
2008年1月の花粉非飛散期の検討から花粉誤認率は、千葉市、成田市ともに0.111で、自動花粉測定器による2008年年2月1日から4月30日の間の従来の自動花粉測定器の測定値とダーラム法による花粉カウント数の相関は、千葉市で0.80、成田市で0.78、一方、新たな補正式を用いた補正では、千葉市で0.76、成田市では0.87といずれも高い値を示した（千葉市のデータは2月に雷による測定器のデータ送信の出来なかった時期があり、3月1日から4月30日までで検討した）。2008年のスギ・ヒノキ花粉飛散は4800個/cm2と平年よりやや多かった。2009年1月の花粉非飛散期の検討から千葉市、成田市の花粉誤認率は0.069,0.101で、自動花粉測定器による2009年2月1日から4月30日の間の自動花粉測定器の測定値（1 ㎥/day）とダーラム法による花粉測定数（1 ㎠/day）との相関は千葉市で0.75、成田市で0.74、補正式を用いた修正値との相関は、千葉市で0.81、成田市で0.77であった。2009年の花粉飛散量は5500個/cm2と大量飛散であった。2010年2月1日から3月31日の間の相関係数は成田市で0.90、千葉市で0.77であり、補正によりそれぞれの相関係数は0.92と0.84に上昇した。2010年の花粉飛散数は千葉市では2100個/cm2と平年の3分の2であったが、成田市では千葉市より多い3300個/cm2であった。さらに、過去の2005年から2007年のそれぞれの年についても検討を行ったが、従来の測定値とダーラムの相関が低い年には新たな補正式で相関値が改善すること、一方、従来の測定値で相関が高い場合には2008年のように、新たな補正式を用いても相関値に大きな変動はみられなかった。
合成ペプチドを用いたELISPOT法によるスギ花粉症患者末梢血リンパ球のスギ花粉特異的Th2メモリークローンサイズは,CD4細胞特異的変化であること、また患者自己樹状細胞を抗原処理して反応させた場合と高い相関がみられ簡便に測定が可能であることが明らかだあった。スギ花粉特異的Th2メモリークローンサイズは1月に比較して5月には1.7-2倍の増加がみられ、スギ花粉特異的IgE値と相関がみられた。ダニアレルギー性鼻炎患者では全例にDer f特異的Th2細胞クローンが確認されたが、1月と5月でそのサイズに変化は見られなかった。

D. 考察：
現状の自動花粉測定器には、空中浮遊粒子との識別に大きな問題がある。これを改善させる方法として、測定全粒子数と花粉測定数の両値の表示が可能な機種を用いて、スギ花粉飛散開始直前でまだ花粉飛散がほとんど見られない時期に地域ごとの花粉誤認率を算定して、機種の花粉見逃し率も含めて新たな補正式を作り補正することで花粉測定の特異性、感度の向上が図れることが明らかになった。ただ、補正式は地域ごとに、毎年異なり、花粉飛散直前に花粉誤認率を測定して作成する必要がある。また、このような形の補正は測定全粒子数と花粉測定数の両値の表示が可能な機種のみに可能で、他の機種には一般化出来ない欠点がある。さらに補正後にも残る自動花粉測定器とダーラム法との花粉数の違いについては、花粉測定器の問題として黄砂の影響を受けること、ダーラム法の問題として突然の降雨でスライドグラス上の花粉が洗われて過小評価になったことなどが考えられた。
合成ペプチドを用いた特異的T細胞クローンの測定は、自己樹状細胞を抗原でパルスして用いる従来の検出法と比較して簡便であり、高い相関もみられた。スギ花粉のみでなく、ダニについても可能である。スギ花粉、ダニそれぞれの特異的T細胞クローンサイズの動態には大きな違いが認められた。

E. 結論
大気中の全粒子の測定値と、その中で花粉と識別した粒子の両方の表示が可能な自動花粉測定器については、花粉見落とし率、花粉誤認率を考慮した新たな補正を加えることで、花粉測定の精度の向上が期待される。抗原ペプチドを用いた特異的T細胞クローンの測定は、比較的少量の採血からELISPOT法により簡便に測定が可能であり、今後、スギ花粉症の発症や寛解、免疫療法のバイオマーカーの検討に関する臨床応用が期待される。

F. 研究発表
1．学会発表
- 堀口茂俊、米倉修二、藤村孝志、岡本美孝. スギ舌下免疫療法の最新の臨床知見. 第50回日本アレルギー学会秋季学術大会、東京、2010年11月.
- 山本陛三朗、米倉修二、堀口茂俊、岡本美孝. 携帯メールを用いた鼻アレルギー症状の登録システムに関する検討.第50回日本アレルギー学会秋季学術大会、東京、2010年11月.

・岡本美孝．アレルギー疾患の予防はどこまで可能か-アレルギー性鼻炎　シンポジウム．日本アレルギー学会秋季大会．2009 年 10 月．秋田．

・岡本美孝、米倉修二、吉江うらら、黒崎元良、堀口茂俊、花澤豊行、高橋雪江、横田匡彦．花粉自動測定器の問題点とその改善に向けての検討。第 47 回日本鼻科学会、名古屋、2008 年 9 月

2．論文発表

・Muradil M, Okamoto Y, Yonekura S, Chazono H, Hisamitsu M, Horiguchi S, Hanazawa T, Takahashi Y, Yokota K, Okumura S. Reevaluation of pollen quantitation by an automatic pollen counter. Allergy Asthma Proc .2010;31:422-427

・岡本美孝、自動花粉測定器を用いたリアルタイム花粉測定とその問題点、臨床免疫・アレルギー科　49；408-414,2008

G.　知的財産権の出願・登録状況（予定を含む）

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

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# The Induced Regulatory T Cell Level, Defined as the Proportion of IL-10$^+$Foxp3$^+$ Cells among CD25$^+$CD4$^+$ Leukocytes, Is a Potential Therapeutic Biomarker for Sublingual Immunotherapy: A Preliminary Report

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**Key Words**
Allergic rhinitis · Biomarker · Foxp3 · Immunotherapy · Interleukin-10 · Japanese cedar · Pollinosis · Regulatory T cell · Sublingual immunotherapy

**Abstract**
***Background:*** Japanese cedar *(Cryptomeria japonica)* pollinosis is one of the most prevalent allergies in Japan. Recently, two reports described the positive effects of sublingual immunotherapy (SLIT) against Japanese cedar pollinosis. However, the therapeutic biomarkers for SLIT are still unclear. We performed this unblinded, nonrandomized, open-label study to identify therapeutic biomarkers for SLIT against Japanese cedar pollinosis. ***Methods:*** We performed an open-label study during one pollinosis season in 2007, enrolling 19 patients from in-house volunteers suffering from Japanese cedar pollinosis. Peripheral blood was obtained from all participants before SLIT treatment as well as before and after the pollen season. The plasma levels of an immunoglobulin specific to a major allergen (Cry j 1) were determined. We analyzed the induction of regulatory T cells (iTregs), namely IL-10$^+$Foxp3$^+$ cells in CD25$^+$CD4$^+$ leukocytes, by flow cytometry. The Th2-type responses were analyzed by cytokine production from peripheral blood mononuclear cells after stimulation with Cry j 1. Clinical symptoms were estimated using a quality of life questionnaire in the middle of the pollen season. ***Results:*** The difference in numbers of iTregs between the medium-only control cell culture and cells stimulated with Cry j 1 was significantly decreased in the non-SLIT group but was unchanged in the SLIT group after the pollen season. The subgroup of the SLIT group with increased iTregs showed more attenuated Th2-type cytokine profiles, and symptom scores in the subgroup with increased iTregs were significantly lower than those in the subgroup with decreased iTregs. ***Conclusion:*** The antigen-specific iTreg level is a potential therapeutic biomarker that correlates with clinical pollinosis symptoms and may be involved in the therapeutic mechanisms of SLIT. 

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## Introduction

Japanese cedar *(Cryptomeria japonica)* pollinosis is one of the most prevalent allergies in Japan; a nationwide survey in 2008 found a prevalence of 26.5% [1]. Compared with the estimate of 13.1% in 2001, this more recent figure implies that the number of affected patients is rapidly increasing [2].

Recently, two reports have described the positive clinical effects of sublingual immunotherapy (SLIT) against Japanese cedar pollinosis [3, 4]. A randomized double-blind study reported that efficacy variable scores in an active treatment group were significantly lower than those in a placebo group, and the quality of life (QOL) symptom score of the active group was almost half that of the placebo group [3]. We also previously reported that active treatment significantly ameliorated symptom scores and symptom-medication scores compared with placebo in a randomized controlled trial. Furthermore, we reported that SLIT decreased the number of Th2 clones specific to CS712, namely recombinant protein-conjugated T cell epitopes from Cry j 1 and Cry j 2 [4, 5].

Several reports have suggested the involvement of Foxp3-positive regulatory T cells (Tregs) in the therapeutic mechanisms of immunotherapy. It was reported that the number of Tregs, namely $CD25^{bright}$ and/or $Foxp3^+CD4^+$ T cells, was significantly increased during specific immunotherapy against bee venom [6]. It has also been reported that $Foxp3^+CD25^+$ and $Foxp3^+CD4^+$ cell levels were significantly increased in the nasal mucosa of patients receiving immunotherapy treatment with grass pollen [7]. mRNA expression of Foxp3 and IL-10 was reported to be induced after SLIT treatment, and the suppression of effector cell proliferation was IL-10-dependent [8]. The central mechanisms by which Tregs downregulate antigen-specific Th2 responses are suggested to be mediated by the production of the suppressor cytokine IL-10 in a soluble or membrane-bound form [9]. However, the therapeutic mechanisms and the relationship between clinical symptoms and Treg induction remain unclear.

In this study, we analyzed Cry j 1-specific Th2 responses and induced Tregs (iTregs), defined as $CD25^+$ $CD4^+$ leukocytes positive for both IL-10 and Foxp3. We considered that antigen-specific iTregs would produce the suppressor cytokine IL-10 with Cry j 1 activation. These Cry j 1-specific iTregs (Cry j 1-iTregs) from the SLIT group were maintained after pollen season, whereas the Cry j 1-iTregs from the non-SLIT group decreased significantly after pollen season. Furthermore, the subgroup of the SLIT group with increased iTregs showed a tendency for attenuated cytokine profiles compared to both the subgroup with decreased iTregs and the non-SLIT group. The subgroup with increased iTregs also showed lower clinical symptom scores than the subgroup with decreased iTregs. We propose that the level of antigen-specific iTregs is a suitable biomarker for the severity of symptoms and the therapeutic effects of SLIT.

## Materials and Methods

*Study Population*

Nineteen in-house volunteers between 22 and 63 years of age, who were otherwise healthy but who had a clinical history of Japanese cedar pollinosis, were enrolled in this pilot study. The diagnosis of Japanese cedar pollinosis was based on clinical history and IgE specific to Japanese cedar pollen of at least class 2 status (CAP-RAST method, Phadia, Tokyo, Japan). Patients who had a history of any immunotherapy, had a current diagnosis of asthma or were pregnant were excluded. The patients in the non-SLIT group were statistically older than those in the SLIT group; however, there was no statistical difference between the groups with regard to the period of suffering from Japanese cedar pollinosis (3–10 years). All patients had showed moderate or severe symptoms in the previous pollen season [10]. Antigen-specific IgE titers for orchard grass, Japanese cypress and house dust mites were also evaluated by the CAP-RAST method. The protocol was approved by the Ethics Committee of Chiba University; written informed consent was obtained from each of the patients prior to participation in the study.

*Clinical Protocols*

Standardized Japanese cedar pollen extract (Torii Pharmaceutical Co. Ltd., Tokyo, Japan) was used for SLIT [11]. The trial was performed from October 2006 to June 2007. The treatment protocol consisted of graded courses of the extract in 50% glycerol, followed by maintenance therapy [4]. The extract was graded in 3 strengths: 20, 200 and 2,000 Japanese Allergy Units (JAU)/ml. The content of Cry j 1 in the 2,000 JAU/ml extract was 1.5–4.2 μg, as determined by enzyme-linked immunosorbent assay (ELISA) and as reported previously [12]. Patients received increasing doses with each vial, beginning with 0.2 ml from the 20 JAU/ml vial and increasing by 0.2 ml a day for 5 days per week; the vaccine was taken sublingually, kept for 2 min without a retention reagent and then spit out. The procedure was then repeated with each vial until the maximum dose (1.0 ml of 2,000 JAU/ml) was reached. The maintenance dose was 1.0 ml of 2,000 JAU/ml once a week until the end of the study. The non-SLIT group was administered neither the vaccine nor a placebo. All participants were allowed to take symptom-reducing drugs.

*Blood Samples*

Peripheral blood was obtained from each patient before the beginning of treatment, before the pollen season and after the pollen season. Peripheral blood mononuclear cells (PBMCs) were isolated from whole peripheral blood by Ficoll density gradient centrifugation using Lymphocyte Separation Medium (MP Bio-

medicals Inc., Solon, Ohio, USA). Isolated cells were counted and tested for viability by trypan blue exclusion prior to culture. The PBMCs were frozen at –80°C and stored in liquid nitrogen using a cell banker (Nippon Zenyaku Kogyo Co. Ltd., Fukushima, Japan) until use.

*Antigens for in vitro Stimulation and ELISA*

Cry j 1 was purified from Japanese cedar pollen according to the method of Yasueda et al. [13] with some modifications. The concentration of purified Cry j 1 was determined by the Lowry method using a detergent-compatible protein assay reagent (Bio-Rad Laboratories Inc., Hercules, Calif., USA). CS712, which is a recombinant protein with 7 conjugated regions of T cell epitopes from Cry j 1 and Cry j 2 [4, 5], was kindly provided by Daiichi Sankyo Co. Ltd. (Tokyo, Japan).

*Antigen-Specific Immunoglobulin Titer*

The Cry j 1-specific IgE titer in the plasma was measured by the method of Yasueda et al. [14]. The Cry j 1-specific IgG4 titer was measured by ELISA as described previously [4].

*Flow Cytometric Analysis*

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 phorbol 12-myristate 13-acetate, 1 μM ionomycin and 2 μM monensin for 6 h. The PBMCs were stained with phycoerythrin-anti-CD25 (eBioscience, San Diego, Calif., USA) and phycoerythrin-Cy7-anti-CD4 antibody (BD Biosciences, San Diego, Calif., USA) in PBS containing 1% FCS and 0.1% sodium azide for 20 min at 4°C. After surface staining, the PBMCs were stained with FITC-anti-Foxp3 (clone PCH101, eBioscience) and allophycocyanin-anti-IL-10 antibody (BD Biosciences) for 30 min at 4°C using a Foxp3 staining buffer set (eBioscience) according to the manufacturer's instructions. The numbers of IL-10$^+$Foxp3$^+$ cells in $10^4$ CD25$^+$CD4$^+$ leukocytes were calculated from the percentage of IL-10$^+$Foxp3$^+$ cells in CD25$^+$CD4$^+$ leukocytes.

*Clinical Symptoms*

The participants were instructed to fill in a QOL questionnaire in the middle of the 2007 pollen season. Japanese cedar pollen scattered from the middle of January to early May in 2007. The Japanese Allergic Rhinitis QOL Standard Questionnaire No. 1 was used for the assessment of QOL symptom scores for allergic rhinitis [15]. The total QOL symptom score was calculated as the sum of each score: none = 0; mild = 1; moderate = 2; severe = 3; very severe = 4. Nasal and ocular symptoms covered by the questionnaire included runny nose, sneezing, nasal congestion, itchy nose, itchy eyes and watery eyes [3].

*Enzyme-Linked Immunospot Assay*

The numbers of IL-4- and IL-5-producing cells stimulated with Cry j 1 or CS712 were determined by enzyme-linked immunospot assay. A 96-well sterile filter plate (Millipore Corp., Billerica, Mass., USA) was coated with monoclonal antibody to human IL-4 or IL-5 (Mabtech AB, Nacka Strand, Sweden), following preincubation with 35% ethanol. After washing with PBS, the plate was preincubated with AIM-V medium at 37°C for 1 h. The medium was discarded, then 3 × $10^5$ PBMCs/well were cultured with fresh medium alone, 10 μg/ml Cry j 1, 20 nM CS712 or 1 μg/ml phytohemagglutinin as a positive control for 17 h at 37°C in AIM-V medium containing 5% human blood plasma fractions. The plates were then washed with PBS, incubated with biotinylated detection monoclonal antibody to human IL-4 or IL-5 for 2 h and then incubated with streptavidin-conjugated alkaline phosphatase for 1 h at room temperature. After washing with PBS, the plates were incubated with BCIP/NBT$^{PLUS}$ (Mabtech AB) for 5 min at 37°C. The numbers of positive spots were automatically calculated by the ImmunoScan™ (Cellular Technology Ltd., Cleveland, Ohio, USA) using the same parameter settings throughout.

*Assay of Cytokine Production from PBMCs*

Isolated PBMCs were cultured at 2.5 × $10^6$ cells/ml with or without 5 μg/ml Cry j 1 for 3 days at 37°C in AIM-V medium containing 5% human AB serum (Sigma-Aldrich Inc., St Louis, Mo., USA). After centrifugation at 300 *g* for 10 min, the supernatant was divided into aliquots and stored at –20°C until cytokine assay. The concentrations of IL-5, IL-10, IL-13 and IFN-γ cytokines were measured by means of the BD™ Cytometric Beads Assay Flex system (BD Biosciences) according to the manufacturer's instructions.

*Data Representation*

The Cry j 1-specific cytokine production and the numbers of Cry j 1-iTregs are presented as the difference between the value from Cry j 1-stimulated cells and that from the medium-only control cell culture. Each upregulation after pollen season was represented as the difference between the pre-pollen season and post-pollen season values.

*Statistical Analysis*

Results are presented as means ± SD. Two-group comparisons were performed using the Wilcoxon t test or Mann-Whitney U test to determine the significance of the difference. p values $<0.05$ were considered significant.

## Results

*Study Population and Adverse Events*

Nineteen adult patients were recruited from in-house volunteers at Chiba University and Chiba University Hospital on the basis of a history of Japanese cedar pollinosis and positive specific IgE (CAP-RAST score over 2; table 1). The 12 subjects who accepted the vaccine were enrolled in the SLIT group, and the 7 individuals who hesitated to take the vaccine were enrolled in the non-SLIT group. Three patients had mild discomfort and 1 patient complained of mild itching in the mouth; however, these adverse events were not serious and did not present a reason to discontinue SLIT.

*Immunoglobulin Production*

The Cry j 1-specific IgE production in the SLIT group was not statistically significantly higher after the pollen

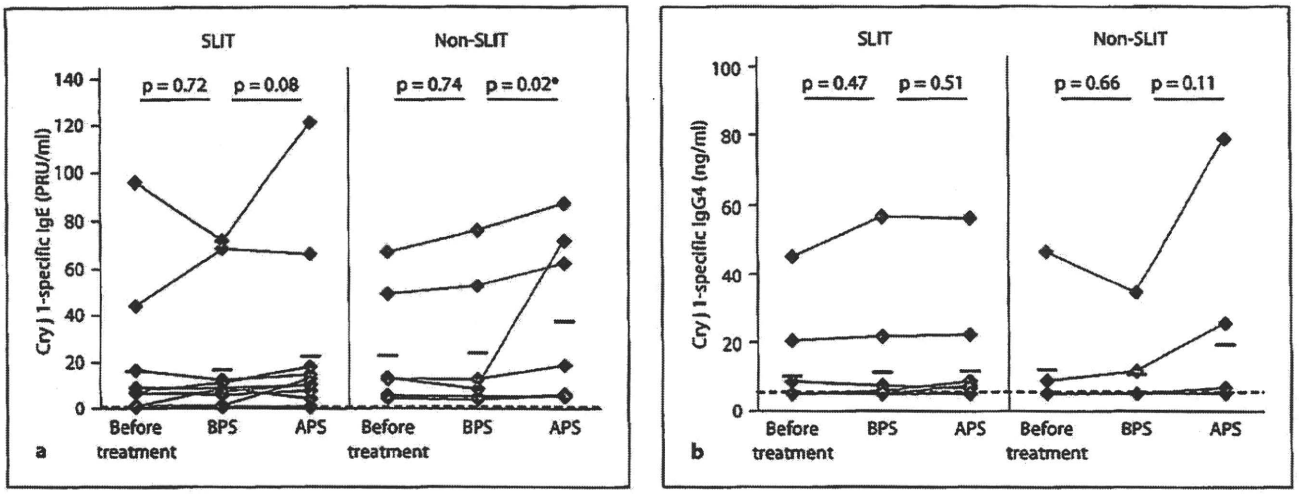


**Fig. 1.** Cry j 1-specific IgE (a) and IgG4 titer (b) from the SLIT and non-SLIT groups before treatment and before (BPS) and after the pollen season (APS). Bars show the group averages. The dashed line indicates the threshold for detection. PRU = Phadebas RAST unit. Statistical analysis was performed using the Wilcoxon t test. * $p < 0.05$.

season compared with before the pollen season ($p = 0.08$); in contrast, IgE production in the non-SLIT group was statistically significantly increased after the pollen season compared with before the pollen season ($p = 0.02$; fig. 1a). Cry j 1-specific IgG4 production was not significantly changed after treatment in either the SLIT or non-SLIT group (fig. 1b).

*Division of the SLIT Group According to the Change in iTregs*

We analyzed a population of IL-10$^+$Foxp3$^+$ cells in CD25$^+$CD4$^+$ leukocytes as a marker of iTregs after stimulation with or without Cry j 1 (fig. 2a). The Cry j 1-iTreg levels, that is, the difference between those stimulated with Cry j 1 and the medium-only control, were significantly increased after treatment, and the difference in numbers of Cry j 1-iTregs before the pollen season was comparable to those after the pollen season in the SLIT group. However, we found that the difference in the non-SLIT group after treatment was comparable with that before treatment and significantly decreased after the pollen season compared to before the pollen season (fig. 2b). The upregulation between before and after the pollen season in the SLIT group (5 ± 42) was higher than that in the non-SLIT group (–24 ± 20), although the difference in the levels between the groups was not statistically significant (fig. 2c).

In all but one participant from the non-SLIT group, the difference in the number of Cry j 1-iTregs was down-regulated after pollen season; in contrast, half of the SLIT group had higher Cry j 1-iTreg levels and the other half had lower levels (fig. 2c). Therefore, we divided the SLIT group into two subgroups according to whether Cry j 1-iTreg levels increased or decreased after pollen season. The total symptom score from the QOL questionnaire in the SLIT group was comparable to that in the non-SLIT group before the division into two groups. After the division, we found that the symptom score in the subgroup

**Table 1.** The characteristics of participants at the time the study started

| | SLIT | Non-SLIT |
|---|---|---|
| Participants | 12 | 7 |
| Sex (M/F) | 9/3 | 5/2 |
| Age, years | | |
| Mean ± SD | 24.1 ± 2.0 | 37.5 ± 15.8 |
| Range | 22–30 | 21–63 |
| IgE class[1] | 3.5 | 4.1 |
| Other allergy[2] | | |
| Orchard grass | 6 | 4 |
| Japanese cypress | 12 | 7 |
| House dust mite | 3 | 5 |

[1] Specific IgE to Japanese cedar pollen; mean CAP allergy class.
[2] Numbers of subjects who had specific IgE of at least class 2.

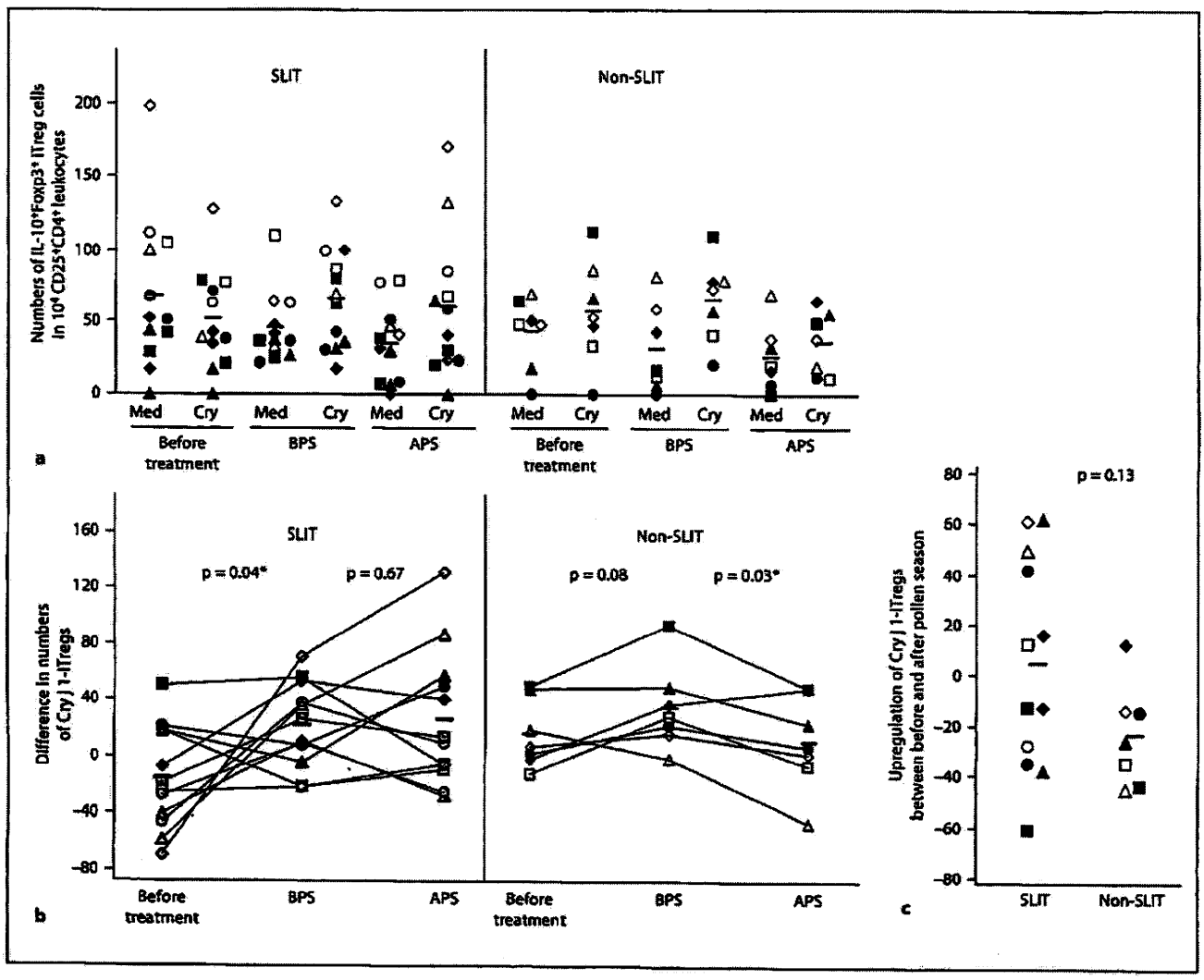


**Fig. 2.** **a** The numbers of IL-10$^+$Foxp3$^+$ cells in $10^4$ CD25$^+$CD4$^+$ leukocytes (iTregs) cultured with (Cry) or without (Med) Cry j 1 before treatment and before (BPS) and after the pollen season (APS). Each symbol in the SLIT group and the non-SLIT group represents an identical individual. Bars show the group averages. **b** The difference in numbers of Cry j 1-iTregs between medium-only control and cells stimulated with Cry j 1 was plotted for before treatment and before and after the pollen season in the SLIT and non-SLIT groups. Bars show the group averages. Statistical analysis was performed using the Wilcoxon t test. **c** The upregulation of Cry j 1-iTregs between before and after the pollen season in the SLIT and non-SLIT groups. Bars show the group averages. Statistical analysis was performed using the Mann-Whitney U test. * $p < 0.05$.

with increased iTregs was significantly lower than that in the subgroup with decreased iTregs ($p = 0.03$; fig. 3).

We also divided the SLIT group into severe and mild subgroups according to their total QOL symptom scores. We found that the upregulation of Cry j 1-iTregs between before and after pollen season in the mild-symptom subgroup was significantly higher than that in both the severe-symptom subgroup and the non-SLIT group (fig. 4).

*Th2-Type Cytokine Profiles*

We analyzed the numbers of Th2-type cytokine-producing cells and cytokine production after stimulation with native Cry j 1. The numbers of Cry j 1-specific Th2-type cytokine-producing cells were analyzed by enzyme-linked immunospot assay after stimulation with Cry j 1 or CS712 (fig. 5a and data not shown). The upregulation of both IL-4- and IL-5-producing cells in the SLIT group

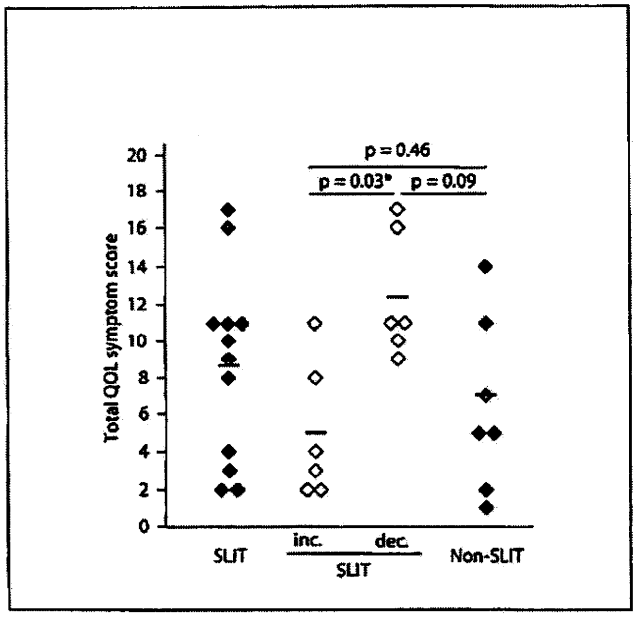


**Fig. 3.** Total symptom score from the QOL questionnaire was plotted for the SLIT and non-SLIT groups as well as for the subgroups from the SLIT group with increased (inc.) and decreased (dec.) iTregs. Bars show the group averages. Statistical analysis was performed using the Mann-Whitney U test. * $p < 0.05$.

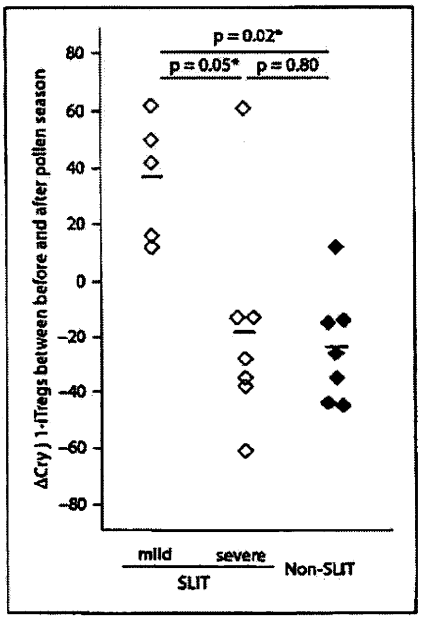


**Fig. 4.** The differences in numbers of Cry j 1-specific iTregs in $10^4$ $CD25^+CD4^+$ leukocytes between before and after pollen season were plotted for the mild and severe subgroups of the SLIT group as well as for the non-SLIT group. The classification into severe and mild subgroups was based on the mean score of the SLIT group. Bars show the group averages. Statistical analysis was performed using the Mann-Whitney U test. * $p < 0.05$.

(IL-4: 29 ± 33; IL-5: 23 ± 28) tended to be attenuated compared with that in the non-SLIT group (IL-4: 54 ± 38; IL-5: 43 ± 28). Furthermore, the upregulation in the subgroup with increased iTregs (IL-4: 19 ± 22; IL-5: 10 ± 11) was much lower than that in both the subgroup with decreased iTregs (IL-4: 40 ± 41; IL-5: 36 ± 35) and the non-SLIT group, although the difference in the levels between the groups was not statistically significant (fig. 5b). The same results were obtained using CS712 for stimulation (data not shown).

Cytokine production was analyzed in culture supernatant after 3 days of culture with Cry j 1 (fig. 6a). The upregulation of Th2-type cytokine production (IL-5 and IL-13), i.e. the differences between before and after pollen season, also tended to be attenuated in the SLIT group (IL-5: 94 ± 126; IL-13: 107 ± 134) compared to the non-SLIT group (IL-5: 178 ± 146; IL-13: 248 ± 222). We found that the Th2 cytokine profile in the subgroup with increased iTregs (IL-5: 54 ± 123; IL-13: 47 ± 110) also showed a strong tendency to be attenuated compared with that in the subgroup with decreased iTregs (IL-5: 134 ± 127; IL-13: 167 ± 137) and was significantly lower than that in the non-SLIT group. The upregulation of IL-13 in the subgroup with increased iTregs showed statistically significant suppression compared with that in the non-SLIT group (fig. 6b). Upregulation of both IFN-γ and IL-10 production induced by Cry j 1 was almost the same in the SLIT (IFN-γ: –1.8 ± 22; IL-10: 0.8 ± 2.2) and non-SLIT groups (IFN-γ: –1.2 ± 32; IL-10: 1.2 ± 2.8; data not shown).

## Discussion

We performed this pilot study to elucidate clinical biomarkers correlated with clinical symptoms in preparation for a future double-blind, placebo-controlled study of SLIT. Only one commercial standardized extract from Japanese cedar pollen is available for clinical use in Japan [11]. The cumulative dose of this major allergen after using the extract for 4 weeks is comparable to 2,500 SQ-standardized grass allergy immunotherapy tablet (SQ-T) in Europe [16]. In spite of the low dose, SLIT against Japanese cedar pollinosis has still been found to effectively

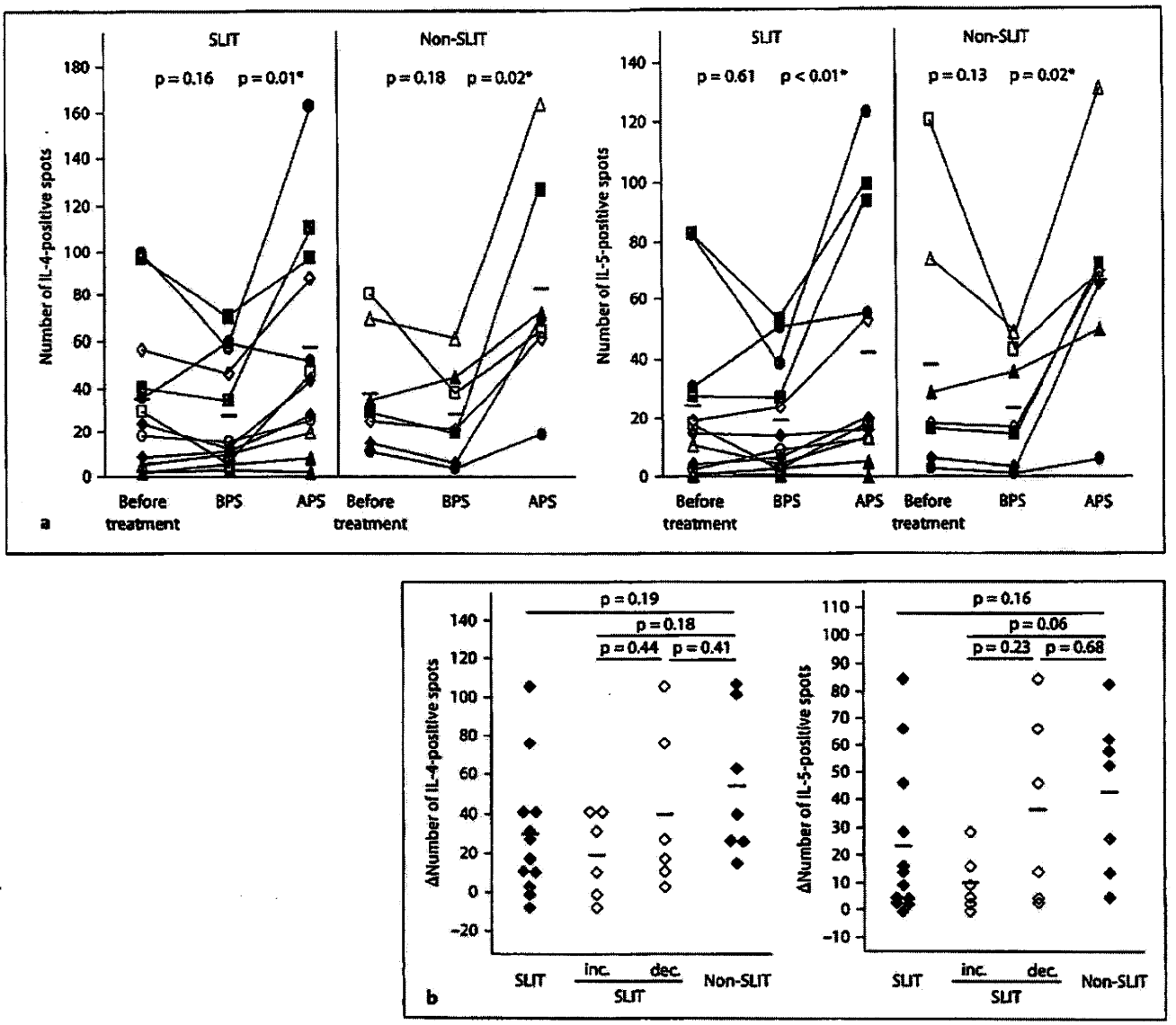


**Fig. 5. a** The numbers of Cry j 1-specific cytokine-producing cells before treatment and before (BPS) and after pollen season (APS). The numbers of positive cells for medium-only control were 1.3 ± 2.4 (SLIT) and 1.5 ± 3.1 (non-SLIT) for IL-4, and 1.0 ± 1.4 (SLIT) and 0.9 ± 1.6 (non-SLIT) for IL-5. Each symbol in the SLIT group or the non-SLIT group represents an identical individual. Bars show the group averages. Statistical analysis was performed using the Wilcoxon t test. * $p < 0.05$. **b** The difference in numbers of Cry j 1-specific cytokine-producing spots is shown as the difference between values before and after the pollen season for each individual from the SLIT, the Cry j 1-iTreg-increased (inc.), the Cry j 1-iTreg-decreased (dec.) and the non-SLIT groups. The numbers of Cry j 1-specific spots were calculated as the difference between the medium-only control and the culture stimulated with Cry j 1.

ameliorate the QOL symptom score, medication score and symptom-medication score [3, 4]. Furthermore, SLIT attenuated antigen-specific Th2 responses and induced iTregs in some patients; this subgroup with increased iTregs showed greater amelioration of the Th2-type cytokine profile and their clinical symptoms.

In this clinical trial, significant induction of Cry j 1-specific IgG4 was not observed in the SLIT group. Our previous report showed induction of Cry j 1-specific IgG4 production with almost the same protocol; the previous study used a piece of bread to retain extract for sublingual administration [4]. The differences in the participants'

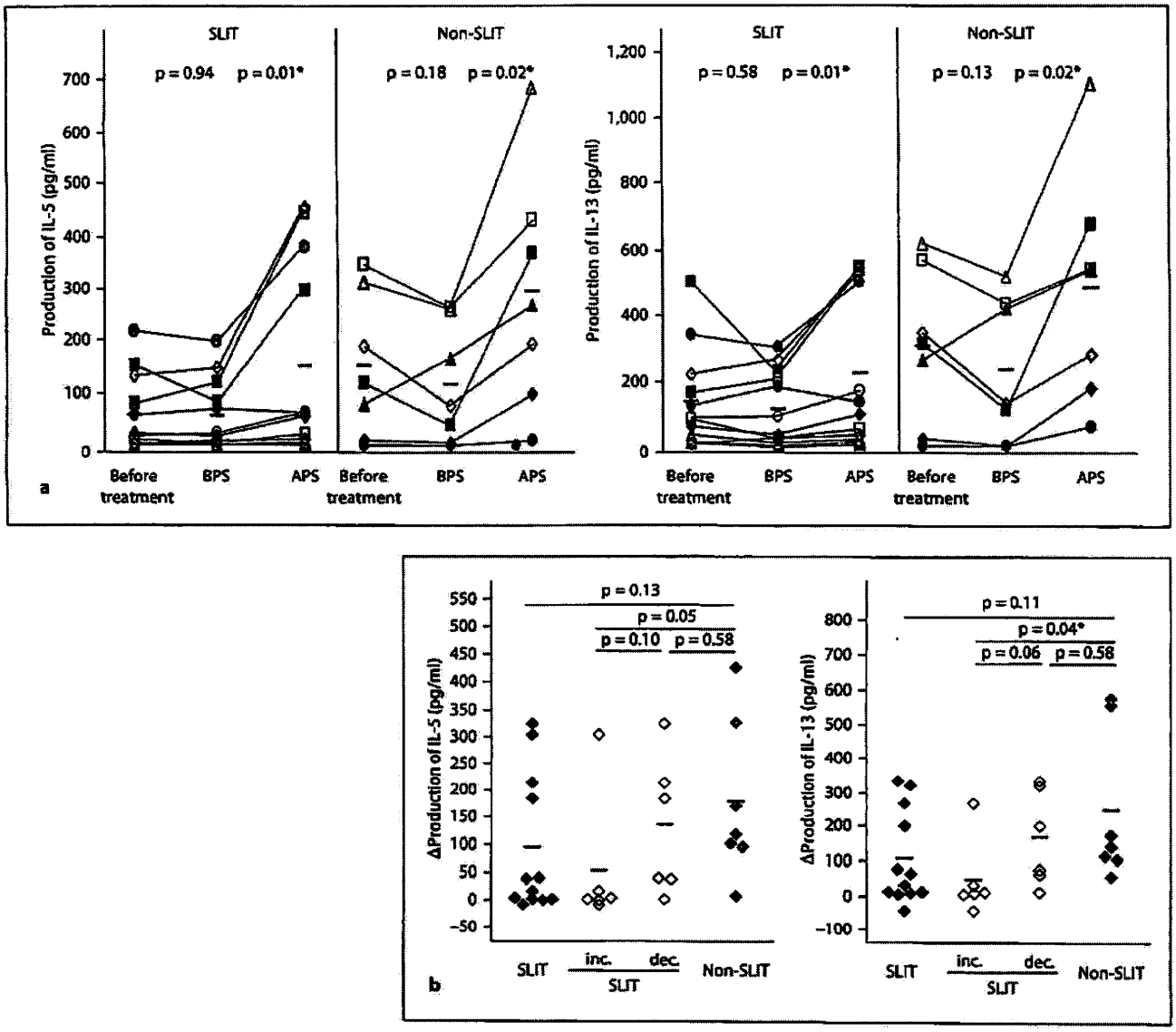


**Fig. 6.** **a** Th2-type Cry j 1-specific cytokine production before treatment and before (BPS) and after (APS) the pollen season. The cytokine production levels for medium-only control were 1.3 ± 2.0 (SLIT) and 3.4 ± 4.5 (non-SLIT) for IL-5, and 6.1 ± 9.6 (SLIT) and 10.6 ± 11.6 (non-SLIT) for IL-13. Bars show the group averages. Statistical analysis was performed using the Wilcoxon t test. * $p < 0.05$. **b** The upregulation of cytokine production induced by Cry j 1 between before and after the pollen season from each individual from the SLIT, the Cry j 1-iTreg increased (inc.), the Cry j 1-iTreg decreased (dec.) and the non-SLIT groups is plotted on the y-axis. The cytokine production induced by Cry j 1 was calculated as the difference between medium-only control and the culture stimulated with Cry j 1. Bars show the group averages. Statistical analysis was performed using the Mann-Whitney U test. * $p < 0.05$.

immunological backgrounds, methods of administration, period of administration and/or the amount of antigen absorbed in the oral mucosa may influence IgG4 induction. Antigen-specific IgG production was reported to be induced by high doses of extract, i.e. 25,000 SQ-T for 18 weeks or 75,000 SQ-T for 8 weeks [16]. This report supports the hypothesis that the amount of antigen adsorbed by the oral mucosa affects the induction of antigen-specific IgG4.

We previously reported that SLIT significantly decreases the clone size of IL-4-producing T cells specific to epitopes from Cry j 1 and Cry j 2 [4]. Also, in the current