D. 考察

アレルギー患者では、アレルゲン特異的なメモリーTh2 細胞が症状の発症に深く関わっている。今回、細胞の生存維持に関与するポリコーム分子に関して解析を行い、メモリーTh1/Th2 細胞形成に関わる重要な機能が明らかになった。このことは、この分子の活性を制御することで、アレルゲン特異的なメモリーTh2 細胞の抑制を通じて Th2 細胞に焦点を当てたアレルギーワクチンの治療法開発の可能性が示唆される。これは新規のストラテジーとして重要な方向性を示すものであるといえる。今後、正常のヒトやアレルギー患者、また代替治療（乳酸菌等プロバイオティクス投与など）を行った患者等においてのメモリーTh2 細胞での RING1B の発現量と機能相関に関して解析したい。また、CD69 分子に関しては、ヒト化抗体の作成に力を注ぎ、この分子をターゲットにした抗体療法の開発と、代替治療を行った患者等において CD69 の発現について解析を行いたい。

E. 結論

メモリーTh2 細胞の形成に重要な分子として RING1B の同定を行った。また、CD69 分子がアレルギー性気道炎症の発症に重要で、この分子をターゲットにした抗体療法の開発の可能性が提示された。代替治療（乳酸菌等プロバイオティクス投与など）を行った患者において、ポリコームや CD69 に焦点を当てたアレルゲン特異的なヒト Th2 細胞の機能修飾の解析の有用性が示唆された。

F. 健康危険情報

特になし

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H. 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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A Randomized Controlled Trial of Sublingual Immunotherapy for Japanese Cedar Pollinosis

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Key Words
Specific Immunotherapy · Allergic rhinitis · Clinical trial · Th2 · T cell clone

Abstract
Background: Japanese cedar pollen represents an important and unique allergen. Sublingual immunotherapy (SLIT) has been suggested to be a highly effective route of desensitization against a variety of allergens. However, little information is available about its use in cedar pollen allergy. ***Methods:*** A blinded randomized, placebo-controlled trial employing SLIT for cedar pollinosis was conducted over a period of 6 months. Sixty-seven subjects were enrolled and the symptom scores during the pollen season were evaluated by a symptom diary, measurement of cedar-specific IgE and IgG4, and determination of Cry j-specific Th2 clones before SLIT and before and after the pollen season. ***Results:*** No major adverse effects were observed in either group. The serum-specific IgG4 activity increased significantly after SLIT in the active group. The active group also exhibited significantly lower symptom scores compared to the placebo. The specific Th2 clone sizes were not significantly different between the groups before the pollen season. However, an increase in the clone size was observed after the pollen season in the placebo group, but not in the active group. ***Conclusion:*** Use of SLIT for Japanese cedar pollinosis was found to be safe and associated with an increase in cedar-specific IgG4 levels. Such therapy inhibited the increase in Cry j-specific Th2 clone size induced by pollen exposure. Finally, use of SLIT resulted in significant improvement of the clinical symptoms of cedar pollinosis in this patient population. These observations suggest that SLIT may offer another safe approach to the management of cedar pollinosis.

Introduction

In recent years, many countries have experienced an increase in the prevalence of allergic rhinitis as well as other allergic disorders [1, 2]. The most important pollen allergens in Japan are tree pollens, such as the Japanese cedar and Japanese cypress [3, 4]. With the exception of the Hokkaido and Okinawa regions, the Japanese cedar is widely distributed and occupies more than 18% of Japan's land surface area. The Japanese cypress is distributed predominantly to the west of the Kanto region. However, the planting of Japanese cedar trees is increasing. Cedar and cypress pollens share a common antigen

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and more than 70% of patients who are allergic to cedar pollen also develop cypress pollinosis [5, 6]. Around the Tokyo region, the cedar pollen season usually starts in the middle of February and is followed by the cypress pollen season which lasts until the beginning of May. Consequently, many patients with cedar pollinosis suffer from heavy pollen exposure for almost 12 weeks. In addition, Japanese cedar and cypress pollen can travel more than 100 km from the source, thereby raining down large amounts of pollen on other large cities. This situation is considerably different from that experienced in other countries where the most common allergens are grass pollens and ragweed, which generally travel distances of several hundred meters, and the pollen season lasts for less than 6 weeks [7]; however, it is similar to Northern European countries and North America where birch and other tree pollens are the major contributors.

Subcutaneous allergen-specific immunotherapy (SCIT) has been evaluated and shown to be an effective approach to change the course of allergic rhinitis [8–13], including Japanese cedar pollinosis [14]. However, an alternative method of administration is still required because the SCIT approach has been associated with the risk, albeit very low, of anaphylactic shock [15] and the inconvenience of frequent visits to the physician's office.

A recent review of randomized controlled studies of sublingual immunotherapy (SLIT) conducted outside Japan has strongly suggested its efficacy against a variety of allergens [16–21]. SLIT could be an attractive approach for Japanese cedar pollinosis if efficacy, safety, mechanisms and effective biomarkers can be clearly established.

The present placebo-controlled randomized studies were designed to determine the effects of SLIT on Japanese cedar pollinosis employing recombinant hybrid peptides consisting of 7 HLA class 2 restricted T cell epitopes of Cry J, the major allergen of Japanese cedar pollen [22].

Methods

Subjects

The study population consisted of 67 patients (33 males and 34 females), ranging in age from 20 to 37 years, who were otherwise healthy, but had a clinical history of Japanese cedar pollinosis for at least the last 3 consecutive cedar pollen seasons. The subjects lived in and around the city of Chiba, where a similar amount of pollen spread would be expected. The diagnosis of cedar pollinosis was based on clinical history, positive allergen-specific skin tests (wheal diameter ≥10 mm) to a standardized cedar pollen extract (Torii Pharmaceutical Co. Ltd., Tokyo, Japan) and serum cedar pollen-specific IgE levels of ≥score 2 by the CAP radioallergosorbent test (CAP-RAST; SRL, Tokyo, Japan). The exclusion criteria included a history of severe asthma, use of antiallergic drugs within 4 weeks and a prior history of any allergen-specific immunotherapy, including therapy for cedar pollen. Pregnant women or those at risk of pregnancy were also excluded. The study was conducted at the Chiba University Hospital and 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 this study.

Japanese Cedar Pollen Extracts

Standardized Japanese cedar pollen extracts (Torii Pharmaceutical Co. Ltd.) were used [23]. The extract [1,000 Japanese Allergy Units (JAU)/ml] contained 1.5 μg of Cry j 1, which is the major allergen of Japanese cedar pollen. The amount of Cry j 1 was quantitated by an enzyme-linked immunosorbent assay, as reported previously [24].

Study Protocol

The study was placebo controlled and single blinded. The enrolled subjects were randomly divided into 2 groups with a ratio of 2:1 according to the table of random numbers by the Department of Pharmacy at the Chiba University Hospital. A controller who was not directly involved in this study was responsible for group allocation. The patients were divided randomly into the active (treatment) and placebo groups. A group allocation number was given to each patient. To prevent the leakage of information, this number was closely guarded jointly by the controller and a member of the ethical committee who was also not directly involved in the study, until accessed with the key after the completion of the study. The active group consisted of 43 patients who received the pollen extract and the placebo group consisted of 24 patients who received the placebo (inactive) for sublingual administration by the spit method (table 1). The sample size was determined based on previous similar studies [25]. The induction/buildup phase was 1 month, with the administration of an increasing daily number of the extract drops at 3 concentrations. The patients received 1 ml of 1,000 JAU extract or placebo once weekly as shown in table 2. Although the safety of the daily administration of SLIT has been reported recently, the weekly administration was chosen in this study in order to further reduce the possibility of any serious adverse events. No study of SLIT for Japanese cedar pollinosis has been reported to date. However, the development of asthma attacks by exposure to pollen has been observed in some patients [14]. The maintenance dose of the antigen in the present SLIT studies was about 100 times higher than that routinely used in SCIT. Administration was started at the beginning of October 2005 and ended at the end of April 2006. The patients carefully completed a pollen diary regarding their nasal symptoms and the usage of rescue drugs (such as antihistamines). Data were collected and analyzed at the Department of Clinical Testing of the Chiba University Hospital.

The nasal symptoms were evaluated on a scale from 0 to 4 in accordance with the *Practical Guidelines for the Treatment of Allergic Rhinitis, Japan* [26], as follows: 0 = no sensation; 1 = mild; 2 = moderate; 3 = severe; 4 = extremely severe. Daily episodes of sneezing and nose blowing were rated 0–4, as follows: 0 = none; 1 = 1–5 episodes; 2 = 6–10 episodes; 3 = 11–20 episodes; 4 = more than 20 episodes. The medications were also recorded according

to drug characteristics and duration of usage, according to the guidelines as follows: antihistamines, mast cell stabilizers and vasoconstrictors were listed as 1, topical ocular or nasal steroids as 2.

Immunoglobulin Assay

Serum Cry j 1-specific IgG4 antibodies were measured using microtiter plates coated with 100 ng/well of Cry j 1 which was purified as reported previously [27]. Allergen-coated wells with serum samples (diluted 1:50) were incubated for 2 h at 37°C, and then washed with PBS. The plates were incubated with 100 μl of biotinylated monoclonal anti-IgG4 antibody (BD Pharmingen; 500 ng/ml) for 1 h at 37°C, and then overnight at 4°C. After washing, the plates were incubated with 100 μl of streptavidin-γ-D-galactosidase conjugate (Roche Diagnostics) at 1:2,000 dilution for 1 h at 37°C, and washed. Finally, 100 μl of 5 mM o-nitrophenyl-β-D-galactopyranoside was added to the wells and incubated for 1 h at 37°C. After the enzyme reaction was stopped with 100 μl of 0.1 M Na_2CO_3, the absorbance at 415 nm was read using a microplate reader.

The specific IgG4 antibody levels were calculated from control curves with serial dilutions of a reference serum pool, which was prepared from 5 sera with high levels of Cry j 1-specific IgG antibody. The IgG4 antibody levels in the reference pool serum were arbitrarily assigned to be 100 U/ml.

Analysis of Th Cytokines and Cell Clones

Peripheral blood mononuclear cells (PBMCs) were obtained by the Ficoll-Hypaque method and stored at −80°C until analysis, using a cell banker (Nippon Zenyaku Kogyo Co. Ltd., Fukushima, Japan).

Th1/Th2 cytokine profiles were analyzed using FACS analysis. PBMCs (5×10^5) were stimulated with PMA and ionomycin for 4 h in the presence of 2 μM monensin, which inhibited the secretion of protein produced de novo. The cells were stained with anti-CD4 antibody for 15 min on ice. After washing with PBS, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized with 0.5% Triton X-100 for 10 min on ice. After blocking with 3% bovine serum albumin for 10 min, the cells were incubated on ice for 30 min with anti-IFN-γ labeled with fluorescein isothiocyanate and anti-IL-4 labeled with phycoerythrin. A flow-cytometric analysis was performed on FACS Calibur (Becton Dickinson, Irvine, Calif., USA). The antibodies for FACS analysis were purchased from BD Bioscience (San Diego, Calif., USA).

The Cry j-specific Th2 clone sizes were determined by an ELISPOT assay using the recombinant hybrid peptide. The hybrid peptide comprised the 7 CD4 T cell determinants of Cry j 1 and Cry j 2, the major Japanese cedar pollen allergens [22]. Almost the entire patient population with Japanese cedar pollinosis respond to this hybrid peptide and the responses are comparable to the individual responses to Cry j 1 and Cry j 2 [22]. The monoclonal antibodies used in the ELISPOT analysis were obtained from Mabtech (Stockholm, Sweden). The anti-human IL-4 or IL-5 monoclonal antibodies were diluted to a concentration of 15 μg/ml in sterile, filtered (0.45 μm) PBS (pH 7.2), and 100 μl per well were added onto nitro-cellulose plates (Millititer; Millipore Corp., Bedford, Mass., USA). The plates were incubated overnight at 4°C and the unbound antibodies were washed with filtered PBS thereafter. After the last wash, PBS was sucked through the membrane under vacuum (Millipore). One hundred microliters of AIM-V medium with or without 20 μM hybrid peptide was added to 5×10^5 cells per well, and the plates were incubated for 10 h at 37°C. All assays were done in duplicate. The cells were subsequently washed before adding 100 μl of the biotinylated monoclonal antibodies (1 μg/ml), and incubated for 2 h at room temperature.

The plates were washed and incubated for 90 min at room temperature with 100 μl of streptavidin alkaline phosphatase (Mabtech) at a dilution of 1:1,000. The unbound conjugate was removed by another series of rinsing before 100 μl of BCIP/NBT substrate solution (Bio-Rad, Richmond, Calif., USA) was added and the plates were incubated at room temperature until dark

Table 1. Baseline characteristics of the patients

	Treatment group (n = 43)	Placebo group (n = 24)
Mean age, years[1]	26.8 ± 5.4	26.4 ± 5.9
Female sex	21 (48.8)	13 (54.2)
Mean duration of cedar pollinosis, years	8.7	9.1
Type of allergic rhinitis		
Cedar pollinosis with perennial	7 (16.3)	3 (17.5)
Cedar pollinosis with other pollinosis	5 (11.6)	4 (16.7)
Cedar pollinosis only	31 (70.5)	17 (70.8)
Additional allergic history		
History of asthma symptoms	2 (4.6)	0
Current asthma symptoms	0	0
History of allergic conjunctivitis	40 (93.0)	19 (75.0)
Cedar pollen RAST score[1]	4.18 ± 1.01	4.14 ± 0.92
Peak of daily total nasal symptoms score in the last cedar pollen season	4.8	4.5

Figures in parentheses are percentages.
[1] Data are means ± SD.

Table 2. Dose and dosing frequency

	Week 1 20 JAU	Week 2 200 JAU	Week 3 2,000 JAU	Week 4 2,000 JAU
Day 1	0.2 ml	0.2 ml	0.2 ml	1.0 ml
Day 2	0.4 ml	0.4 ml	0.4 ml	
Day 3	0.6 ml	0.6 ml	0.6 ml	
Day 4	0.8 ml	0.8 ml	0.8 ml	
Day 5	1.0 ml	1.0 ml	1.0 ml	
Day 6				
Day 7				

The induction phase with an increasing number of extract drops over 5 days a week at 3 concentrations for 3 weeks and the maintenance phase (week 4) with 1 ml of 1,000 JAU extracts once weekly are shown.

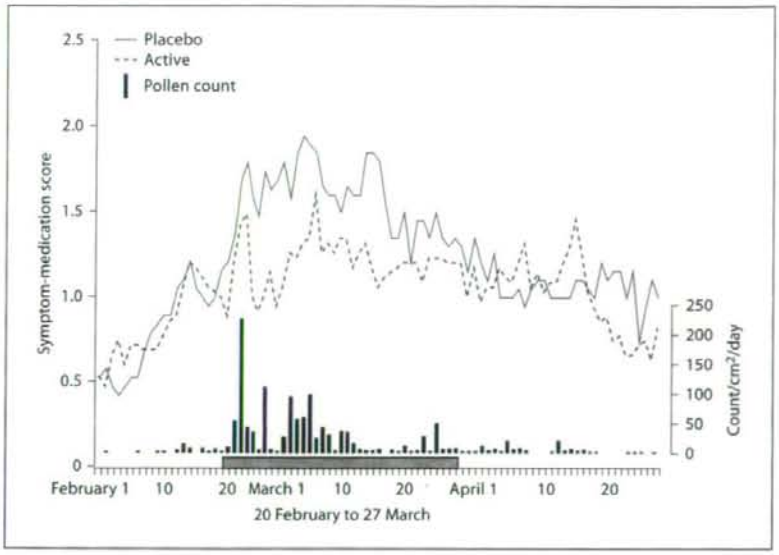
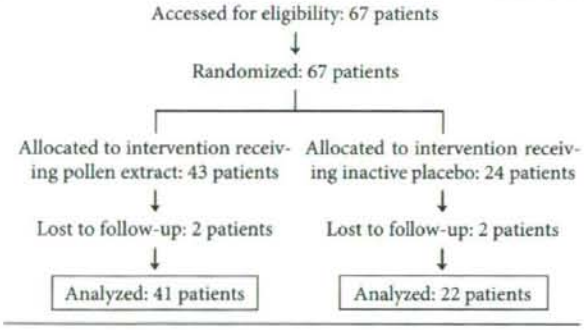


Fig. 1. Daily combined Japanese cedar and cypress pollen counts in 2006 in Chiba measured by the Durham pollen sampler and symptom-medication score (mean values) of patients during the pollen season.

Table 3. Study participation



spots emerged (1 h). The color development was stopped by repeated rinsing with tap water. After drying, the spots were captured photoelectrically and counted by a computed analysis to avoid any visual bias, using an auto counter (ImmunoScan; CTL, Cleveland, Ohio, USA).

Statistical Analysis

After completion of the study, the clinical and laboratory data were analyzed by a biostatistician who was not involved in carrying out the clinical trial. After completing the analysis, the allocation identification numbers for the active and placebo groups were accessed with a key. The Mann-Whitney U test was performed to compare symptom scores as well as symptom-medication scores between placebo and active groups. The Wilcoxon signed rank test was used for paired comparisons of the Cry j 1 specific IgG4 levels before and after SLIT. All statistical analysis was performed using the GraphPad Prism software, version 4.

During the statistical calculations in the present studies, the β error was defined as 0.2, power was 80% and the α error was defined as 0.05. Values of $p < 0.05$ were considered statistically significant.

Results

Four patients were withdrawn from the study for personal reasons, but not due to any adverse effects. All other subjects exhibited full compliance with the study protocol. As a result, 63 patients (41 patients from the active group and 22 patients from the placebo group) were analyzed further for effectiveness of SLIT (table 3).

Adverse Effects

Fifteen adverse effects were reported during the treatment. Of these, 13 subjects were in the active treatment group and 2 in the placebo group. Two patients in the active group complained of mild urticaria of the face or breast. The remaining subjects exhibited mild oral pruritus or oral pain (Common Terminology Criteria for Adverse Event grade 1). All adverse effects were transient and resolved spontaneously. No intervention was necessary.

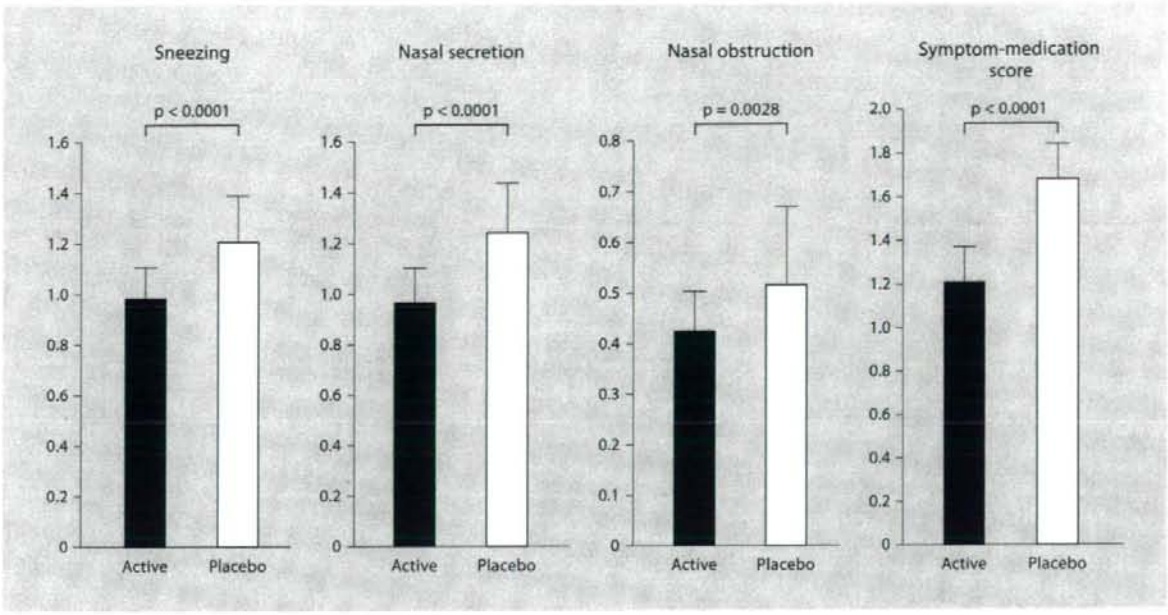


Fig. 2. Average symptom scores of sneezing, nasal secretion, nasal obstruction and symptom-medication score during the high pollen season, from 20 February to 27 March 2006. The average score of the active group was significantly lower than that of the placebo group.

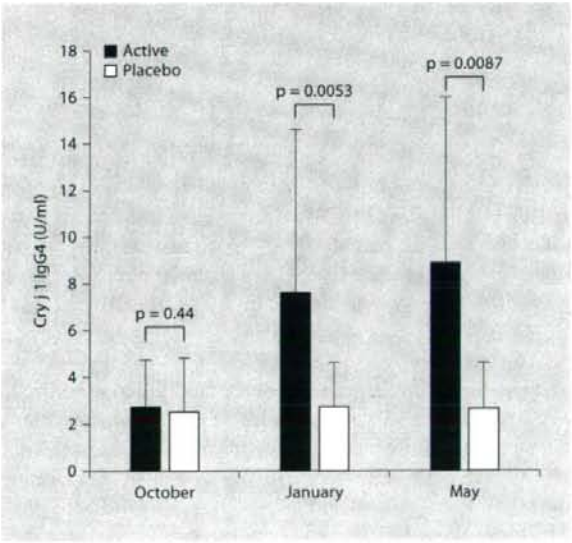


Fig. 3. Levels of serum Cry j 1-specific IgG4 before/after pollen dispersal. Specific IgG4 significantly increased in the active group but not in the placebo group and a significant difference was observed between the groups ($p < 0.05$).

Pollen Counts

In 2006, the Japanese cedar pollen season started in the middle of February and was followed by cypress pollen, which continued until the end of April. The duration of the pollen season extended from 20 February to 27 March. The combined annual amount of cedar and cypress pollen was 1,154/cm^2 according to the Durham pollen sampler in Chiba.

Symptoms

The nasal symptoms and medication scores during the pollen season are shown in figures 1 and 2. The temporal profiles of nasal symptoms and medication scores were in general similar in the active and placebo groups and reflected the pollen counts in the community. However, the scores were lower in the active (treatment) group, especially during the peak of the pollen season as shown in figure 1.

The symptom scores for sneezing, nasal secretion volume, degree of nasal obstruction and medication scores were significantly higher in the placebo group compared to the active (treatment) group ($p < 0.01$) during the peak of the pollen season as shown in figure 2.

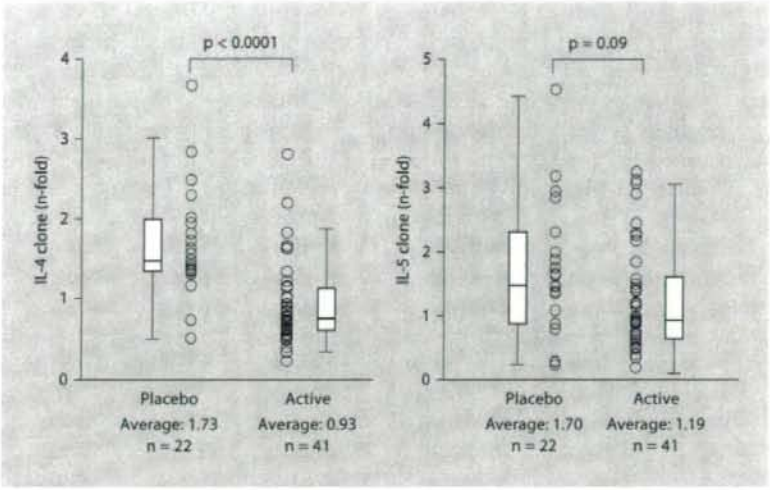


Fig. 4. Relative change of Cry j-specific IL-4 and IL-5 clone sizes in May, after the pollen season, compared with those in January before the season.

Serum Immunoglobulin

There were no significant differences in the 2 study groups for the Japanese cedar pollen-specific IgE and IgG4 levels in the serum samples collected in October, just before SLIT was initiated. On the other hand, after the initiation of immunotherapy, Cry j-specific IgG4 levels exhibited a significant increase in the active group in January before the pollen season. Significantly higher levels of specific IgG4 were observed in the active group for at least 4 months after the initiation of the immunotherapy as shown in figure 3. No significant effects of immunotherapy were detected for the levels of specific IgE (data not shown). No changes in the specific IgE levels were observed relative to the cedar pollen dispersion between January and May. The levels of specific IgG4 also did not exhibit any change after pollen exposure in both the active and placebo groups (fig. 4) and the levels did not correlate with the nasal symptom scores (data not shown).

Th1/Th2 Cytokine Profiles

The number of Th1/Th2 cells in the peripheral blood CD4 T cells did not change significantly and no significant difference was observed between the 2 groups during the study period (data not shown).

Cry j-Specific Th2 Clone Sizes

The number of Cry j-specific IL-4 and IL-5 spots showed a strong correlation. Although the number of spots was similar between the active and placebo groups before the pollen season, a significant increase in IL-4 spots was observed only in the placebo group after the pollen season. The increase in IL-4 clone size during the pollen season in the active and placebo groups was 1.71 $\pm$ 0.71 and 0.70 $\pm$ 0.52 (mean $\pm$ SD), respectively ($p < 0.0001$). On the other hand, the increase in IL-5 clone size between the active and placebo groups was not significant, the power ($1 - \beta$ error) was 0.58 ($p = 0.09$) as shown in figure 4.

Discussion

Although the use of SCIT has been found to be safe for immunotherapy for a variety of pollen allergies, the practical inconvenience associated with its use prompted this study to explore alternative routes of administration. A recent review of randomized controlled studies of SLIT has suggested both its efficacy and safety [16–21]. Although SLIT for Japanese cedar pollinosis is an attractive alternative route, no randomized controlled studies have been carried out to date. The observations of particular importance reported here have shown that the use of SLIT significantly increased the levels of pollen-specific IgG and downregulated the size of pollen-specific Th2 lymphocyte subset clones.

In order to avoid adverse effects, such as local pain and swelling associated with injection and possible anaphylactic reactions, a dose of 40 JAU/month as a maintenance dose has generally been utilized in SCIT for Japanese pol-

linosis. In this study 1,000 JAU/week (4,000 JAU/month) was used as a maintenance dose in SLIT, which was 100 times more than that used in SCIT. The choice for such a dose was somewhat arbitrary and the optimal dose required for effective and safe use of SLIT remains to be determined.

The combined Japanese cedar and cypress pollen counts generally exceed 3,000/cm^2, as measured by the Durham pollen sampler in Chiba and Tokyo. However, in 2006 the pollen counts were 1,154/cm^2, which was one third of the average for the last 5 years. In Japan, the pollen counts and the counts/cm^2 are usually measured by the Durham samplers, which utilize a gravimetric method that is different from the Burkard sampler, a volumetric method that is widely used in European countries. Direct comparison of the counts by the 2 methods can be difficult, because the ratio between the 2 methods depends on the local meteorological conditions and the types of pollen. When these methods were compared in 2005, the counts obtained by the Burkard sampler were about 12 times higher than those obtained by the Durham sampler [28].

During SLIT, no adverse effects greater than Common Terminology Criteria for Adverse Event grade 3 were observed. Three months after SLIT, serum Cry j 1-specific IgG4 was elevated in the active group. However, the specific IgE levels were not significantly different between the groups.

Several previous studies employing SLIT have observed an increase in allergen-specific IgG4 levels and specific IgG4/IgE ratios in the serum [29, 30]. However, the precise role of increased IgG4 in the effectiveness and outcome of such immunotherapy remains to be determined. Lima et al. [31] reported that the IgG levels correlated with the clinical efficacy as a blocking antibody, but other studies have failed to demonstrate such a correlation [32]. The increased levels of Cry j 1-specific IgG4 antibody in this study indicate that SLIT can induce specific antibody responses. However, the role of IgG4 antibody in the mechanisms of clinical effectiveness remains to be defined. No relationship between the IgG4 responses and the clinical efficacy was observed in this study.

In the present studies, the use of SLIT was associated with milder clinical symptoms and lower medication scores during the pollen season and a significant reduction in each symptom was observed. As pointed out earlier, the doses in this SLIT study were much higher than those generally used in SCIT. However, in SLIT with other allergens, the clinical efficacy has been shown to be allergen dose dependant [33]. Although the swallow-SLIT method is currently widely used, we selected the spit-SLIT method to further reduce the possibility of adverse effects, since no SLIT trials with Japanese cedar pollinosis have been carried out to date. Further studies will be needed to assess the dose responses, temporal intervals and vehicles of administration to obtain optimal effectiveness with SLIT.

Cedar pollen-specific IgE and IgG4 did not increase significantly with pollen exposure, which might be explained in part by the relatively small amount of pollen dispersal observed during these studies.

The total number of Th2 cells in the peripheral blood did not increase during the pollen season and Th1/Th2 cytokine profiles did not change throughout the study in either group. However, the profiles of the allergen-specific Th cells were quite different. The patients with cedar pollinosis are thought to have cedar pollen-specific memory Th cell clones and the treatment is aimed at diminishing the size of Th2 clones. Since the Th cell response is restricted in MHC class 2, it is necessary to use a class 2 restrictive T cell epitope to measure the reaction of T cell clones in response to the allergen. Japanese cedar-specific IL-4 and IL-5 producing memory T cell in the peripheral blood were examined by an ELISPOT assay using Japanese cedar pollen-specific peptides. Although the number of cedar peptide IL-4 and IL-5 T cells was low, all patients exhibited specific spots, ranging from 5 to 100 spots/10^5 PBMCs. The number of cedar pollen-specific Th2 cells did not correlate with the cedar pollen-specific serum IgE nor IgG4 levels. This may be related to the possibility that IgE and IgG4 synthesis is controlled by many other factors, including Th1 cells and memory B cells.

The size of the cedar pollen-specific Th2 cell clones was not different between the active and the placebo groups before the pollen season. Interestingly, these Cry j-specific Th2 clone sizes were increased about 1.7-fold during the cedar pollen season by pollen exposure in the placebo group. However, this increase was not observed in the active group and SLIT suppressed the increase in specific Th2 clone sizes. The change in the clone size did not correlate with the levels of allergen-specific IgG4 antibody.

Several recent studies have explored the significance of regulatory T cells in allergic and autoimmune disorders [34–37]. The suppression of allergen-specific Th2 clones observed in this study may be a reflection of such regulatory T cells, although the precise contribution of different T cell subsets remains to be examined.

In summary, this study has demonstrated that SLIT for Japanese cedar pollinosis was safe and is associated with increased cedar pollen-specific IgG4. Such therapy also inhibited the increase in specific Th2 lymphocyte clone sizes induced by the exposure to cedar pollen. It is also suggested that the use of SLIT appears to be an acceptable alternative to SCIT for Japanese cedar pollinosis.

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Clinical and Experimental Allergy, **38**, 405–412

ORIGINAL PAPER **Asthma and Rhinitis**

Seasonal changes in antigen-specific T-helper clone sizes in patients with Japanese cedar pollinosis: a 2-year study

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Clinical and Experimental Allergy

Summary

Background Allergic rhinitis (AR) is a typical type I allergic disease that occurs through the induction of allergen-specific effector T cells. Once established, new effector T cells derive mostly from memory T cells that are capable of surviving for extended periods, although the mechanisms by which these memory functions are maintained have not yet been clarified. In particular, the exact life-span of memory T cells is still not well understood.

Objective Pollinosis patients seemed to be suitable subjects to investigate because such patients are exposed to antigens strongly for only a limited period once a year. We compared the seasonal changes in memory T-helper type 2 (Th2) between pollinosis and perennial allergic subjects.

Methods The clone sizes of the Japanese cedar pollen-specific memory Th cells were measured by an ELISPOT assay using specific peptides from the patients with cedar pollinosis, and the seasonal changes were noted. This study was performed for 2 years. The cedar-specific IgE levels in the peripheral blood were also studied. Mite allergy patients were also enrolled in the study.

Results The Japanese cedar-specific IL-4-producing Th2 cells were detected in all patients examined, although the number of cells was low. These Th memory cells increased during the pollen season and decreased during the off-season. However, more than 60% of the cedar-specific memory Th2 cells survived up to 8 months after the pollen season. The cedar-specific IgE levels exhibited changes similar to the cedar-specific Th cells. On the other hand, there was no drifting of Th memory clone size with the mite allergics, and the IgE levels also did not change.

Conclusions While pollen-specific Th cells decreased after pollen exposure, their memory functions continued. Memory clone size maintenance therefore requires repetitive antigen irritation.

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Introduction

In recent years, many countries have experienced an increase in the prevalence of allergic rhinitis (AR) [1, 2]. In Japan, Japanese cedar (*Cryptometria japonica*) and Japanese cypress (*Chamaecyparis obtusa*) pollens are considered to be the major unique allergens and their extent of dispersal is quite large, travelling more than 100 km and thus causing serious pollinosis [3, 4].

Pollinosis is thought to be an adaptive immune response that manifests as a type I allergic reaction, and it occurs as a consequence of fundamental allergenic mechanisms involving the induction of pollen-specific T-helper type 2 (Th2) effector cells from naïve Th0 cells [5]. Most effector T cells are short-lived, but few effector T cells become long-lived memory T cells. Once a memory T cell is established, it retards the induction of new effector T cells from naïve Th0 cells, according to the principle of the 'original antigenic sin'. Hence, most effector T cells are derived from memory T cells [6–10]. This concept describes a phenomenon in which the antibody response elicited in an individual after a secondary viral infection reacts more strongly to the viral variant that originally infected the individual. A similar phenomenon

is likely to be observed in Th cell responses as they play critical roles in promoting antibody responses.

Patients with type I allergy are thought to have allergen-specific memory Th cell clones. Immune-therapeutic intervention directed at diminishing the size of these clone memory Th2 cells and shifting the cytokine type of memory Th clones is thought to play a considerably important role in finding a complete cure.

However, the mechanism by which the antigen-specific Th memory clone is maintained in an allergic patient has not yet been clarified. There are several reports describing the peripheral blood to be re-stimulated by a specific antigen, while the Th2 cytokine response was measured both in season or/and out of season [11–13] as well as before and after immunotherapy [14–16]. In those studies, peripheral blood mononuclear cells (PBMCs) were stimulated by an antigen, and measured Th2 cytokine or its mRNA. However, those cytokine responses that were re-stimulated by whole antigen reflected various kinds of cells such as T cells, B cells, macrophages and antigen-presenting cells and did not precisely reflect the function or the exact number of antigen-specific Th cells. Because the Th cell response is restricted in major hitocompatibility complex class II, it is necessary to use the Th cell epitope of class II restrictive to measure the reaction only for Th cell clones respond to allergen. Our purpose is to estimate the duration of life-span of allergen-specific memory T cells. We therefore directly examined the number of specific Th2 cells to respond to class II restrictive T cell epitope, which matched with Japanese human leukocyte antigen (HLA) variation. These kinds of studies have not yet been carried out. Pollinosis seemed to be a suitable subject to investigate the life-span of Th memory clones because patients are exposed to the antigen for only a limited period. In the present study, we examined the specific memory clone size, which is a population size of memory T cells that recognizes the same specific HLA restrictive epitope and produces isologous cytokine. We tried to detect IL-4 productive cells using only seven T cell epitopes; as a result, the total summation of these seven kinds of clones is the clonal size.

The limited seasonal nature of antigen exposure is useful for elucidating the mechanisms used in the maintenance of Th memory clone sizes. We examined the Japanese cedar pollen-specific Th clone sizes and the associated seasonal changes in patients with cedar pollinosis, while also comparing the yearly change in the clone size due to pollinosis with that due to perennial mite allergies that were detected by 14 T cell epitopes.

Materials and methods

A total of 41 patients with Japanese cedar pollinosis were enrolled in this study. The ages of the 20 males and 21 females ranged from 20 to 51, with an average of 31.1 years. The diagnosis of Japanese cedar pollinosis was based on the occurrence of typical nasal symptoms during the cedar pollen season and the detection of Japanese cedar-specific IgE by CAP-RAST (score: 2 or more). All patients had symptoms for at least 3 years. None of the patients received immunotherapy or immunosuppressive drugs (including steroids) within 8 weeks before the start of the study. The study received prior approval from the Ethics Committee of the Chiba University (Chiba, Japan). A written, witnessed informed consent was obtained from all patients. The study design is shown in Table 1. From 2003 to 2004, 23 patients participated in this study, and from 2004 to 2005, another 18 patients were enrolled. Twenty-two patients with perennial AR due to mite were also enrolled in this study. The ages of the 10 males and 11 females with perennial AR were from 20 to 48, with an average of 28.8 years. The diagnosis of mite allergy was based on the occurrence of typical nasal symptoms and the detection of Der f IgE by CAP-RAST (score: 2 or more).

Ten healthy subjects were also enrolled as controls. They were all negative for symptom episodes and allergen IgE.

The blood samples were collected every 3 months after July, and the PBMCs were obtained by the Ficoll–Hypaque method from the patients. The samples were stored in liquid nitrogen until analysis.

Clinical symptoms

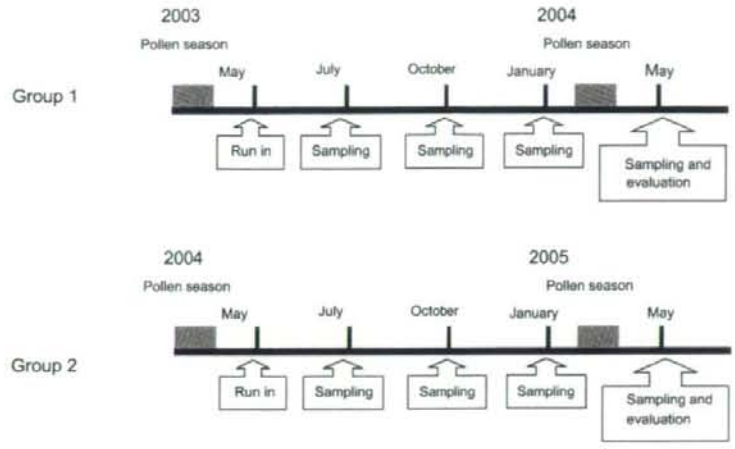
The nasal symptoms were evaluated on a scale from 0 to 4 in accordance with the practical guidelines for the treatment of AR [17], as follows: 0, no sensation; 1, mild; 2, moderate; 3, severe; and 4, extremely severe. Daily episodes of sneezing and nose blowing were rated 0 to 4, as follows: 0, none; 1, 1–5 episodes; 2, 6–10 episodes; 3, 11–20 episodes; and 4, >20 episodes. The medication was also recorded according to drug characteristics and duration of usage, according to the guidelines, as follows: antihistamine, mast cell stabilizers and vasoconstrictor were 1, and topical ocular or nasal steroids were 2.

Reagents

Antibodies. The monoclonal antibodies (MoAb) used for the ELISPOT assay were acquired from MABTECH (Stockholm, Sweden). For coating, MoAb 82-4 and MoAb 1-DIK were used to coat human IL-4 and IFN-γ, respectively. For detection, MoAb 12-1 and 7B6-1 were used to detect human IL-4 and IFN-γ, respectively. For the FACS analysis, anti-human Cy5-conjugated CD4, FITC-conjugated IFN-γ and PE-conjugated IL-4 were purchased from Dako (Tokyo, Japan).

Peptides. A recombinant hybrid peptide was used for the ELISPOT assay. This peptide comprised of the seven CD4 T

Table 1. Time schedule for examination



The Group 1 patients were recruited after the pollen season of 2003 and the Group 2 patients were recruited after pollen season of 2004. The blood sampling was performed every 3 months starting in July. The last blood sampling was performed after the pollen season of May, and the samples were analysed simultaneously.

cell determinants of Cry j 1 and Cry j 2, and the major Japanese cedar pollen allergens. Almost all the patient populations responded to this hybrid peptide, which is comparable to the response to Cry j 1 and Cry j 2. Moreover, because the seven peptides do not contain IgE-binding residues of *C. japonica* allergens, the recombinant peptide will not directly influence IgE-bearing cells such as mast cells, basophils and B cells [18]. The recombinant peptides for mite allergy were also prepared. This peptide comprised of the 14 CD4 T cell determinants of Der f 1 [19] and Der f 2 [20], and the major mite allergens. Almost all the patient populations responded to these peptides, which is comparable to the response to Der f 1 and Der f 2. Those peptides were Class II restricted and recognized Th cells only.

ELISPOT

The ELISPOT assay was performed according to the manufacturer's instructions. Briefly, the anti-human IL-4 or IFN-γ MoAbs were diluted to a concentration of 15 μg/mL in sterile, filtered (0.45 μm) PBS (pH 7.2), and 100 μL/well was added onto nitro-cellulose plates (Millititre, Millipore Corp., Bedford, MA, USA). The plates were incubated overnight at 4 °C and the unbound antibodies were washed with filtered PBS thereafter. After the last wash, the PBS was sucked through the membrane under vacuum (Millipore Corp.). One hundred microlitres of the pre-stimulated cell suspension was added to each well in duplicate, and the plates were incubated for 10 h at 37 °C. The cells were subsequently washed before adding 100 μL of the biotinylated MoAbs (1 μg/mL), and were then incubated for 2 h at room temperature. The plates were then washed and incubated for 90 min at room temperature with 100 μL of streptavidin alkaline phosphatase (Mabtech, Stockholm, Sweden) at a dilution of 1 : 1000. The unbound conjugate was removed by another series of rinsing before 100 μL of BCIP/NBT substrate solution (Bio-Rad, Richmond, CA, USA) was added, and the plates were incubated at room temperature until dark spots emerged (1 h). The colour development was stopped by repeated rinsing with tap water. After drying, the spots were captured photo-electrically and counted by a computed analysis to avoid any visual bias, using Auto Counter (ImmunoScan, CTL, Gmünd, Germany).

Pollen counts

The combined annual cedar and cypress pollen counts were measured using Durham pollen samplers.

Statistical analysis

Wilcoxon's paired rank sum tests were used to compare the mean values. A Friedman two-way ANOVA was used to

analyse paired data. Peason's tests were used to determine the correlation coefficients.

Results

The cedar and cypress pollen counts determined using the Durham samplers were 470 cm/season in 2004, which was 1/20 of the average for the last 10 years. In 2005, we obtained 7852 cm/season, which was threefold higher than that of the average for the last 10 years. The seasonal symptoms were comparatively mild in 2004, and comparatively serious in 2005. The mean symptom-medication scores during the pollen season in 2004 and 2005 were 1.4 ± 2.1 and 2.8 ± 2.3 (mean $\pm$ SD), respectively.

Before the study, we compared the peptide-specific Th2 clone size of the patients with that of healthy controls in October, which is off pollen season. After stimulation with 10 nmol/L of cedar-specific peptides, the mean values of the IL-4 spot from the healthy controls and patients with cedar pollinosis that were obtained are shown in Fig. 1. Positive spots were obtained only in the samples from the pollinosis patients, but not from controls (A), although spots were equally obtained in the samples from both patients and controls when stimulated by Con A as a pan T cell stimulant (B). We also confirmed peptides for mite. The IL-4 spots were obtained only in the samples from the patients, but not from the controls (Fig. 2a), although spots were equally obtained in the samples from both patients and controls when stimulated by Con A as a pan T cell stimulant (Fig. 2b). Peptide-specific IL-4 spots were detected in all samples examined. Approximately 10–100 peptide-specific IL-4 spots were observed in the wells

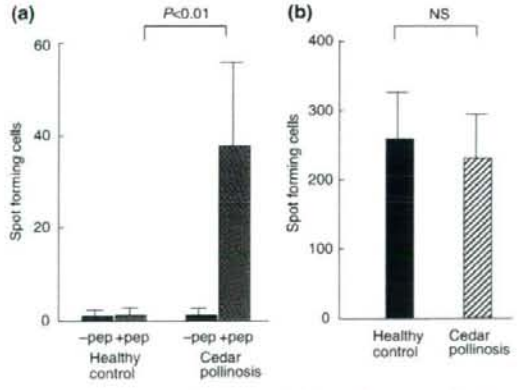


Fig. 1. The mean values of IL-4 spots in the samples from the healthy control subjects and patients with cedar pollinosis are shown. (a) 1×10^5 cells were stimulated by a peptide derived from Cry j 1 and Cry j 2. The spots were obtained only in the samples from the pollinosis patients, but not from the controls. (b) 1×10^4 cells were stimulated by Con A, a pan T cell stimulant. The spots were equally obtained in the samples from both the patients and the controls. NS, not significant.

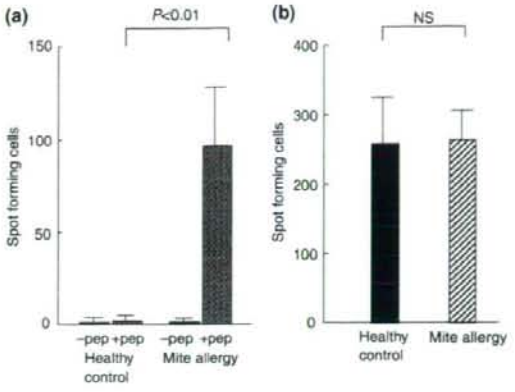


Fig. 2. The mean values of IL-4 spots in the samples from the healthy control subjects and patients with cedar pollinosis are shown. (a) 1×10^5 cells were stimulated by a peptide derived from Der f 1 and Der f 2. The spots were obtained only in the samples from the pollinosis patients, but not from the controls. (b) 1×10^4 cells were stimulated by Con A, a pan T cell stimulant. The spots were equally obtained in the samples from both the patients and the controls. NS, not significant.

incubated with 10^5 PBMC based on the ELISPOT assay. These spots were analysed in triplicate in each of the experiments and exhibited good reproducibility.

We carried out a depletion assay in advance with whole PBMC from three patients with Japanese cedar pollinosis using an antibody-conjugate magnetic bead kit (MACS system, Miltenyi Biotec GmbH, Tokyo, Japan). When CD4 was depleted, the spots of ELISPOT disappeared; however, after the depletion of CD8, the number of spots was equal to that with whole PBMC. The depletion of CD28 also caused the spots to disappear. These results show that the present ELISPOT assay using a hybrid peptide was CD4 restricted and the CD28 expression was indispensable for the detection of the spots (data not shown).

The seasonal changes in peptide-specific IL-4 spots are shown in Fig. 3. The IL-4 spots decreased after July, and were at their lowest in January before the onset of the cedar pollen season, which were almost 60% of those observed in July. The IL-4 spots increased during the cedar pollen season in 2004 despite the small amount of pollen (Fig. 3a). The same trend in seasonal changes was obtained during the 2005 season. The IL-4 spots decreased after July, and were at their lowest in January before the onset of the cedar pollen season, which were almost 60% of those observed in July (Fig. 3b). Interestingly, the clone size on May 2005 was 40% larger than that on July 2004. This phenomenon was not seen during the 2004 season.

The seasonal changes in the cedar-specific IgE levels in the serum of the patients are shown in Fig. 4. While the cedar-specific IgE decreased after the pollen season in 2003, it did not increase during the cedar pollen season in

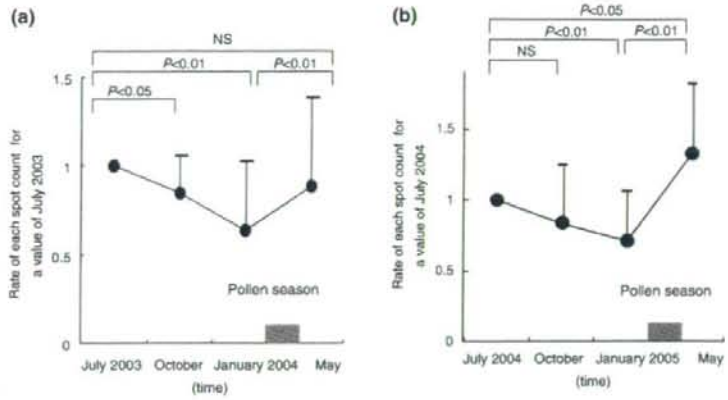


Fig. 3. The samples of each time-point were analysed simultaneously. The Cry j 1 and 2 peptide-specific IL-4-producing cells were counted using the ELISPOT technique. The rate of each spot count for the value in July is shown. (a) Twenty-three patients were enrolled during the 2004 season, which was characterized by very little pollen scattering. (b) Eighteen patients were enrolled during the 2005 season, which was characterized by massive pollen scattering. NS, not significant.

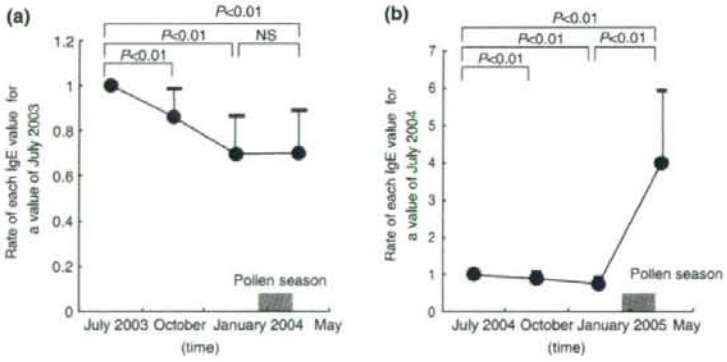


Fig. 4. The samples of each time-point were analysed simultaneously. Cedar-specific IgE were analysed using the RAST technique. The rate of each IgE for the value in July is shown. (a) Twenty-three patients were enrolled during the 2004 season, which was characterized by very little pollen scattering. (b) Eighteen patients were enrolled during the 2005 season, which was characterized by massive pollen scattering. NS, not significant.

2004 (Fig. 4a). The specific IL-4 spots decreased during the pollen season in some patients, and the cedar-specific IgE levels decreased during the pollen season in all patients. During the 2004–2005 season, the cedar-specific IgE levels decreased after the pollen season in 2004, but increased fivefold during the pollen season in 2005 (Fig. 4b). The number of cedar-specific IL-4 spots did not show a correlation with the cedar-specific IgE levels.

The yearly changes in the mite-specific IgE levels and Der f peptide-specific IL-4 spots were also examined in the samples from 22 patients with mite allergy. The yearly changes in mite-specific IgE and mite-specific memory Th2 clone size could not be clearly obtained (Fig. 5).

The patients with mite AR had persistent nasal symptoms all year around. Although the symptom scores varied among the patients as well as the seasons, the deviation was occasionally large; overall, no significant difference was observed. The pollinosis only demonstrated significant symptoms during the pollen dispersal season; however, the patients' nasal symptom scores during the pollen season were higher than those of the mite AR patients as shown in Fig. 6.

Discussion

We examined the Japanese cedar-specific IL-4-producing memory T cells in the peripheral blood of patients with