trial, SLIT attenuated Cry j 1-specific cytokine production, the numbers of IL-4- and IL-5-producing cells and IL-5 and IL-13 production in culture supernatant (fig. 5b, 6b). The difference in downregulation was not statistically significant between the SLIT and non-SLIT groups, but the results showing this tendency were reproducible. No significant difference in Th2-type cytokine production after stimulation with Cry j 1 was observed after sublingual administration of extract alone (i.e. the cytokine levels before and after pollen season in the SLIT group; fig. 5a, 6a). SLIT may attenuate the upregulation of antigen-specific Th2-type responses activated through natural exposure in pollen season. Therefore, the amount of scattering pollen may influence the degree of amelioration of Th2 responses by SLIT.

The upregulation of Cry j 1-specific iTregs, that is, the difference in levels of Cry j 1-iTregs between before and after pollen season, is suggested to be a suitable biomarker for clinical symptoms and therapeutic effects. The average difference in numbers of Cry j 1-iTregs after pollen season, that is, the difference in numbers of IL-10$^+$Foxp3$^+$ cells in CD25$^+$CD4$^+$ leukocytes between those stimulated with Cry j 1 and the medium-only control, was 24.8 in the SLIT group and 9.3 in the non-SLIT group in 10^4 CD25$^+$CD4$^+$ leukocytes after 3 days' culture with Cry j 1 (fig. 2b). The number of antigen-specific Tr1 is reported to be estimated at 0.5–10 in 10^4 whole peripheral CD25$^+$CD4$^+$ Tregs [17]. That estimate suggests the appropriateness of the numbers of Cry j 1-iTregs reported in this study. The subgroup with increased iTregs showed more attenuated Th2 cytokine profiles and a lower total QOL symptom score than the subgroup with decreased iTregs and the non-SLIT group (fig. 3, 5b, 6b). Several reports have shown that Foxp3-positive cells and/or IL-10-producing cells are induced by immunotherapy and that IL-10 is crucial for downregulation of inflammatory Th2 responses [6, 8, 18, 19]. Foxp3-expressing CD4$^+$CD25$^+$ cells are reported to be induced in the nasal mucosa after immunotherapy, and local induction of iTregs is suggested to be important to suppress local inflammation during pollen season [7]. Furthermore, the basal frequencies of Tregs defined as CD4$^+$CD25^{bright}Foxp3$^+$ cells in the peripheral blood of patients with severe allergic reactions to insect stings were reported to be lower than in individuals without a history of allergic diseases, and the Treg population was upregulated to a level comparable to that of nonallergic subjects after immunotherapy against bee venom [6].

The QOL symptom score was higher in the group with decreased Cry j 1-iTregs than in the group with increased Cry j 1-iTregs. IL-17-secreting Foxp3$^+$ Treg cells were recently identified in humans, and IL-17 mRNA expression was significantly correlated with poor clinical outcome after SLIT [20–22]. A low dose of SLIT may induce IL-17-secreting Tregs rather than IL-10-secreting Tregs for nonresponder populations and thereby worsen clinical symptoms.

We divided the SLIT group into two subgroups according to whether IL-10 or Foxp3 single-positive cells increased or decreased after pollen season. However, groups with IL-10 or Foxp3 single-positive cells showed no difference in Th2 cytokine profiles or symptom scores among the increased, decreased and non-SLIT groups (data not shown). We hypothesize that a population of Foxp3 or IL-10 single-positive cells may include many antigen-nonspecific Tregs and effector cells, whereas only antigen-specific iTregs are available as a therapeutic biomarker. These data suggest that IL-10 and Foxp3 double-positive cells rather reflect the antigen-specific iTregs that could be used as therapeutic biomarkers of SLIT.

Soluble IFN-γ and IL-10 production in culture supernatant was not upregulated by the SLIT treatment and did not differ between the SLIT group and the non-SLIT group (data not shown). This suggested that SLIT did not induce Cry j 1-specific Th1-type responses and that the membrane-bound form of IL-10 may be more important than soluble IL-10 for downregulation of Cry j 1-specific Th2 cells. Further investigation is needed to clarify the induction of Th1 cells and IL-10-mediated suppressive mechanisms by iTreg cells. In this trial, we failed to detect other regulatory molecules at the protein level, such as TGF-β from culture supernatant or cytotoxic T lymphocyte-associated protein-4 and glucocorticoid-induced TNF receptor on the surface of CD4$^+$ T cells (data not shown). Further investigations are needed to analyze these regulatory molecules at the mRNA level. We are currently undertaking a transcriptome analysis of CD4$^+$ cells from the SLIT and non-SLIT groups after stimulation with Cry j 1. On the other hand, the regulatory mechanisms of peripheral human Tregs were suggested to occur in a cell contact-dependent but cytokine-independent manner [23]. Furthermore, this report suggested that the regulatory function of human Tregs was independent of CD28, cytotoxic T lymphocyte-associated protein-4, TGF-β and IL-10 [23]. In order to elucidate the therapeutic mechanisms of SLIT, analysis of the regulatory function of iTregs is also important.

In this paper, we investigated antigen-specific iTregs as a therapeutic biomarker for SLIT. However, this study was a preliminary open-label study with a small population; therefore, a randomized, double-blind, placebo-controlled study of a large population will be needed to evaluate iTregs as a therapeutic biomarker for SLIT.

Acknowledgments

We sincerely thank Drs. Takashi Saito and Yasuyuki Ishii (RIKEN, Kanagawa, Japan) for their helpful comments and the fruitful discussions. This work was supported in part by a grant from the Ministry of Health, Labor and Welfare in Japan and in part by the Global Centers of Excellence program entitled Global Center for Education and Research in Immune System Regulation and Treatment (Ministry of Education, Culture, Sports, Science and Technology, Japan).

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REVIEW ARTICLE

Present Situation of Cedar Pollinosis in Japan and its Immune Responses

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ABSTRACT

Recent observations have suggested significant worldwide increase in the prevalence of allergic rhinitis and cedar pollinosis. 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. Cedar pollinosis is a typical type 1 allergic disease by an adaptive immune response that occurs through the induction of allergen-specific effector T cells from naïve T cells. We examined the number of Japanese cedar pollen specific memory Th cells in the peripheral blood of the patients and found that the cedar pollen specific IL-4-producing Th2 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. Natural killer T(NKT) cells represent a unique lymphocyte subpopulation and their activity is not restricted to MHC antigens. NKT cells play an important role in innate immunity, however, the participation in development of allergic rhinitis could not be clarified.

KEY WORDS

cedar pollinosis, cedar specific Th memory cell, epidemiology, natural killer T cell

CEDAR POLLEN

In recent years, many countries have experienced an increase in the prevalence of allergic rhinitis.[1,2] Dust mite allergen is responsible for at least 90% of cases of perennial allergic rhinitis, while arboreal pollen, including that of cedar and Japanese cypress, is important in Japan.[3,4] Cedar forest covers nearly 18% of the total land area of Japan, while Japanese cypress is concentrated in the Kanto region and the western part of the country. Both cedar and Japanese cypress produce enormous amounts of pollen. In Japan, pollen counts are typically measured using the gravimetric method with a Durham sampler, in contrast to Western countries in which a Burkard sampler is typically used. In a study in Chiba Prefecture in 2005, the amount of air-borne pollen counted with a Burkard sampler was about 12 times greater than that counted with a Durham sampler.[5] In addition, distinct from grass pollen, which only spreads less than 100 meters, cedar and cypress pollen travel a long distance and reach major cities, including Tokyo and Osaka, causing wide-spread pollinosis, although no actual data describing the distance traveled was available. A detailed simulation study considering the results of real-time pollen distributing information was conducted using large computers and Figure 1 shows the source and areas from which the cedar pollen detected at Chiba University Hospital had spread. These dark spots indicate the areas where the cedar pollen originated. Pollens blow to Chiba city from the cedar planting areas of Boso Peninsula, as well as from the north Kanto area, Nikko, Izu Peninsula and Shizuoka Prefecture. This study suggests that cedar pollen actually can travel more than 100 km and cause pollinosis in a large area.

Cedar pollen dispersal precedes Japanese cypress pollen dispersal, and approximately 70% of patients with cedar pollinosis are also allergic to Japanese cypress pollen because of a common antigen.[6] Dispersal of cedar and Japanese cypress pollen generally exhibits an arch-shaped pattern with time: cedar pollen dispersal starts in early February and reaches a peak between late February and early March, and is fol-

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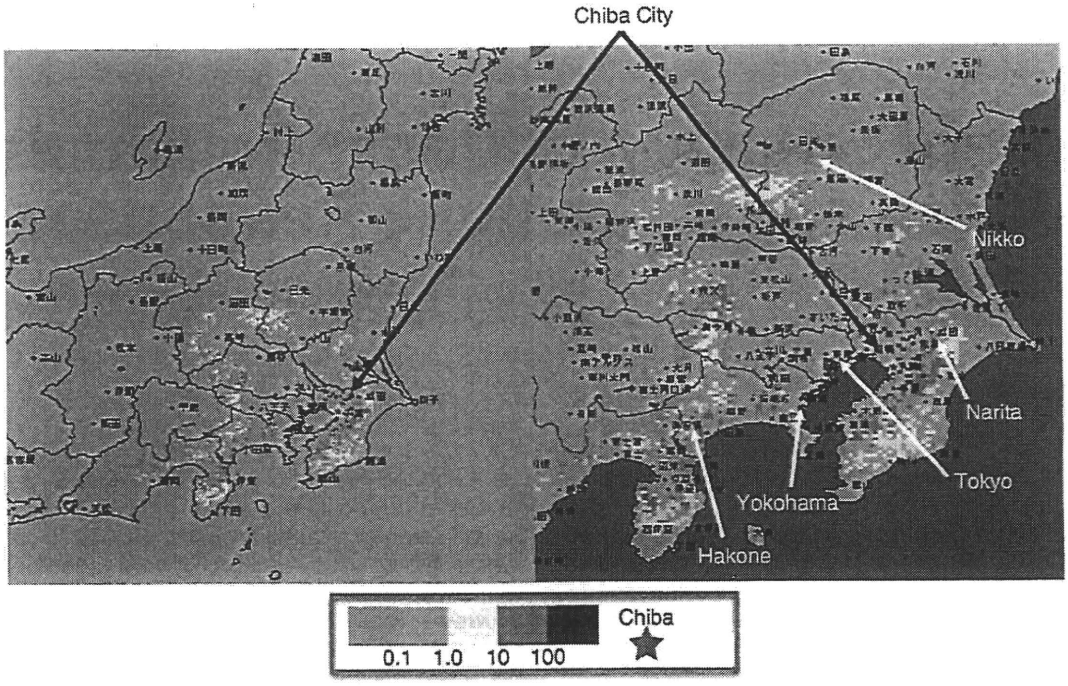

Fig. 1 The source areas from which the cedar pollen detected at Chiba University Hospital spread. This is the computer simulation study done by Mr. Kunihiko Yokota *et al.*, at Weather Service Co.,Ltd..

lowed by dispersal of Japanese cypress pollen, which reaches a peak from late March to early April, with some variation due to changes in the climate each year.[7,8] The pollen dispersal season lasts for more than 10 weeks in and around the Tokyo area.

PREVALENCE OF CEDAR POLLINOSIS IN JAPAN

A survey based only on a questionnaire has the risk of inclusion of a high rate of false-positive cases, because allergic rhinitis is sometimes difficult to distinguish from acute upper respiratory infection and even normal healthy individuals may exhibit mild, nonspecific nasal symptoms, such as sneezing and nasal secretion. In particular, cedar pollen dispersal season is also high flu season. An allergen-specific IgE test is necessary to avoid a high incidence of false positives, but it has been difficult to conduct an epidemiological study in Japan because of laws preventing use of personal information. In 2008, a questionnaire was posed to the Otorhinolaryngologists nationwide to determine whether their families suffered from allergic rhinitis. Although the rate of return of the questionnaire was low, i.e., 40% and the bias of the population could not be ignored, an accurate diagnosis was expected.

According to the analysis of this questionnaire,[9] the prevalence of perennial allergic rhinitis and of cedar pollinosis was 23.4% and 26.5%, respectively. In particular, the prevalence of cedar pollinosis increased more than 10% compared with that observed in a similar questionnaire conducted in 1998. Although the peak of cedar pollinosis is in those in their thirties to forties, the age onset of pollinosis has been decreasing (Fig. 2).

Figure 3 shows the annual amount of cedar pollen dispersal in Japan, which we examined in 2005. The darker brown parts indicate areas where cedar pollen counts were high. We studied the influence of various amounts of pollen exposure on the development of pollinosis and mite allergic rhinitis in elementary school students from schools in rural areas where the movement of students out of or into the school was uncommon. The annual amount of cedar and cypress pollen differed among these five regions. The pollen level was very high in southern Yamanashi: about 7,000/cm^2 on average for the last five years, as determined using Durham pollen samplers. In contrast, the pollen level was low in northern Yamanashi and inland Akita, at about 2,000/cm^2, and very low in coastal Akita, at about 500/cm^2. The pollen level in Chiba was about 4,000/cm^2.

Figure 4 shows the detection rate of cedar- and mite-specific IgE in students in these regions. The positive rate for Japanese cedar was about 60%, except for students in coastal Akita, who had a rate of only 23%. The positive rate for mite IgE was about 50% in each region. These results suggest that the sensitization rate for mite allergen is almost the same nationwide, whereas that for cedar pollen is depend-

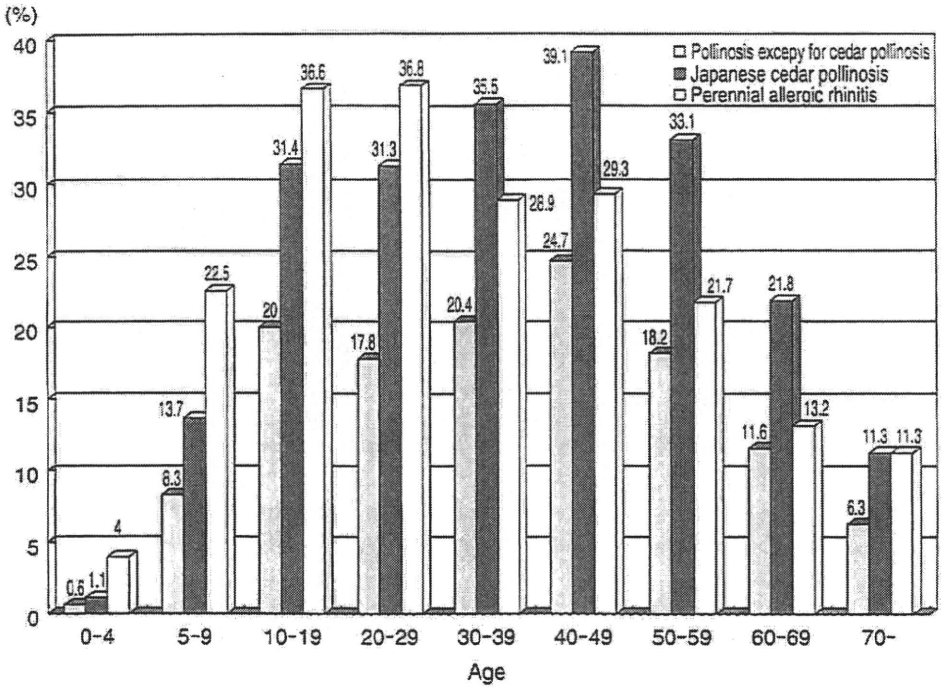

Fig. 2 The prevalence rate of allergic rhinitis in Japan in 2008 (from reference 9).

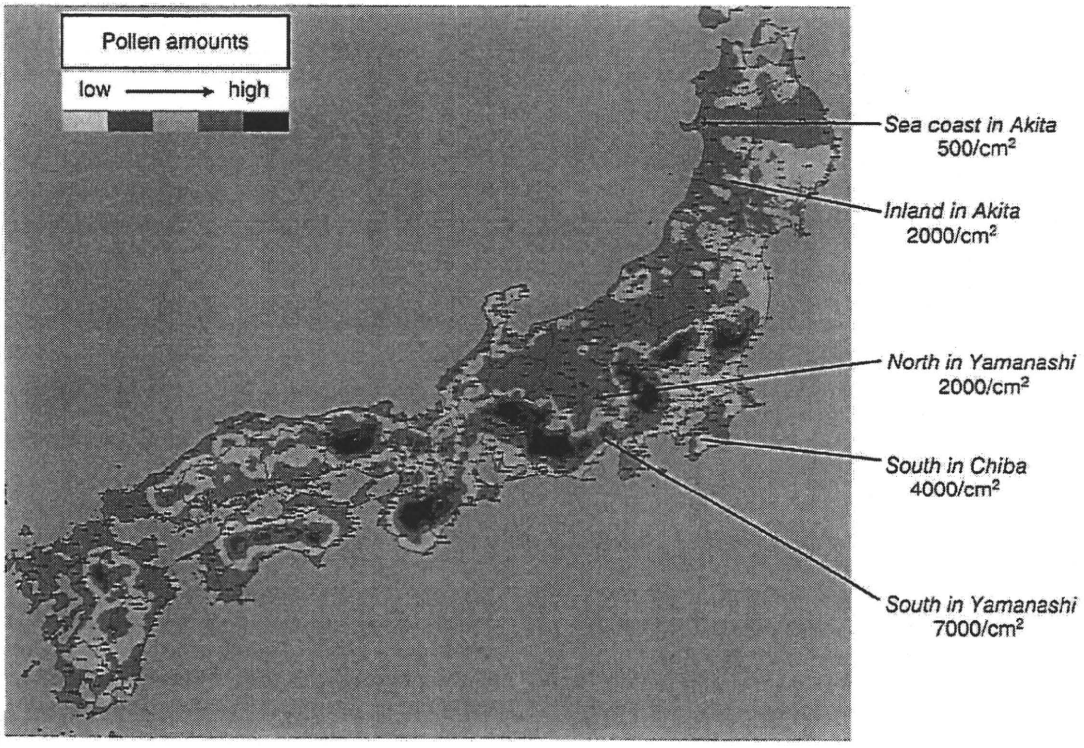

Fig. 3 Annual amount of cedar and cypress pollen dispersal in Japan in 2005.

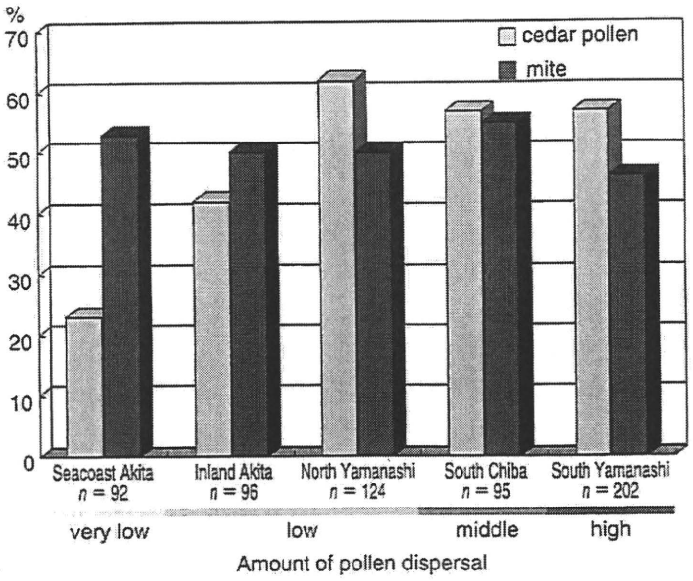

Fig. 4 The detection rate of cedar and cypress pollen-specific IgE in all 4th and 5th grade students in the elementary schools.

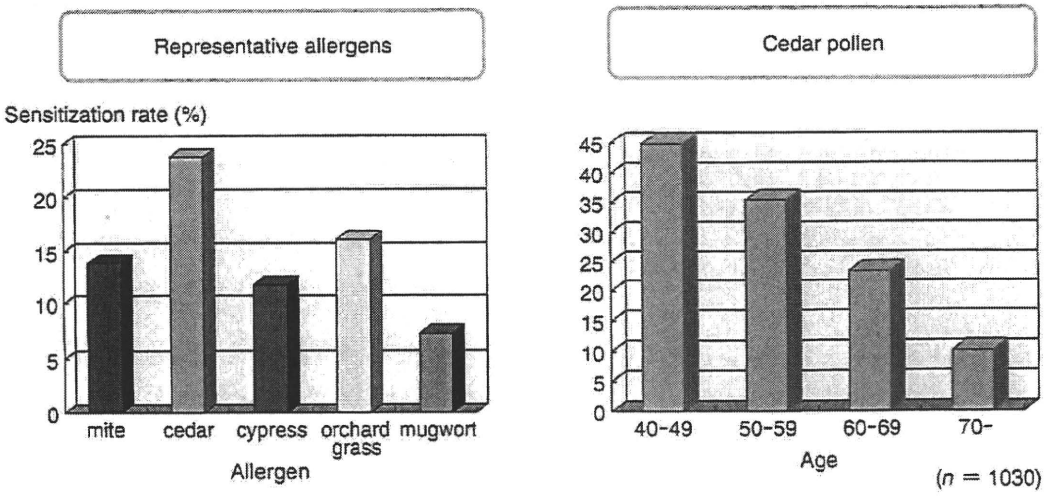

Fig. 5 The sensitization rate to the representative allergen and age distribution of cedar pollen-specific IgE in the adult residents in the forties to seventies in the rural small town in South Chiba.

ent on pollen counts. A very low level of pollen results in a low rate of detection and allergen avoidance is undoubtedly important for prevention. However, a high rate of allergic sensitization can be induced by a relatively small amount of pollen, and it is likely to be very difficult to reduce the amount of pollen exposure to a level that will prevent sensitization. Furthermore, tolerance was not easily induced in students in southern Yamanashi who had been receiving high pollen exposure every year since birth. Interestingly, the incidence of mite allergic rhinitis and pollinosis in these sensitized students was almost the same; about 30 to 35% in each region, respectively.

We have also undertaken medical examination of middle-aged adult residents in their forties to seventies in a rural small town (Maruyama-cho) in South Chiba every year since 1995.[10] The examination includes responses to a questionnaire and testing for specific IgE in serum using a CAP-RAST system. Figure 5 shows the sensitization rate to the representative allergens and the age distribution of cedar pollen-specific IgE. Deterioration of cedar-specific IgE is ob-

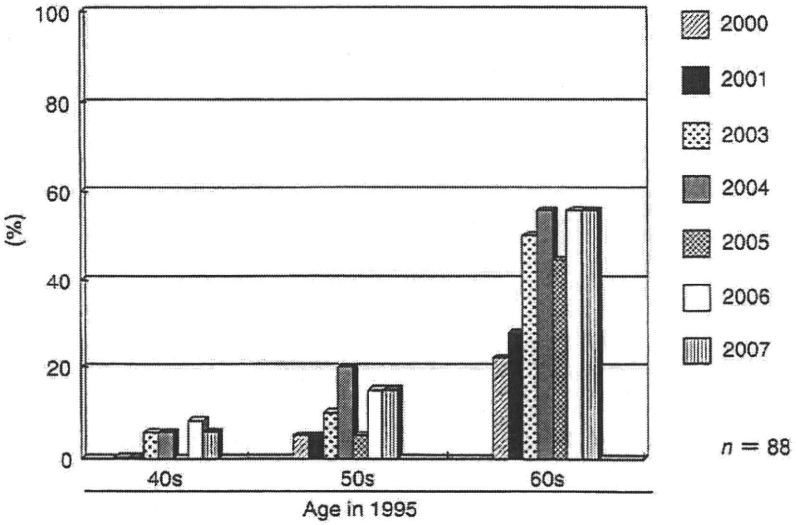

Fig. 6 The rate of change to negative over the last 13 years in cedar pollen-specific IgE in the residents who had tested positive for anti-cedar pollen specific IgE in 1995 and then had received examination every year.

served in elderly subjects. Figure 6 shows the rate of change to negative over the last 13 years in cedar pollen IgE in residents who had tested positive for anti-cedar pollen IgE in 1995. The IgE assays were performed at the end of each cedar pollen season. It appears that the IgE titer is affected by the spread of pollen each year. Interestingly, however, the negative change for 13 years is not commonly observed even in their forties to fifties. The rate of the cedar pollinosis determined by clinical symptoms in combination with positive cedar pollen IgE has also not decreased among these aged subjects.

THE LONG-TERM COURSE OF PATIENTS WITH ALLERGIC RHINITIS

One hundred and seventy-seven patients who were treated in our department from 1970 to 1995 consented to undergo a detailed re-examination. A comparison between the recent symptoms and those observed 10 to 30 years ago showed that 30% of adult patients exhibited some improvements and 10% had resolution. However, only 20% of the pediatric patients exhibited mild improvement of symptoms, whereas the remaining had the same or even worse symptoms as those in childhood (data not shown: in preparation for submitting). Regarding the allergen-specific IgE, a change to negative was not observed in any patients with cedar pollinosis and was seen in only a few of the mite-allergic patients. Thus, natural resolution is not commonly observed in allergic rhinitis and most pediatric patients grow to adulthood without natural improvement of symptoms.

CEDAR POLLEN SPECIFIC MEMORY T CELLS

It has been suggested that dysregulation of cytokine synthesis from Th1 and Th2 cells is fundamental to the pathogenesis of allergic diseases. However, no significant difference was observed between the two groups in the Th1/Th2 cell profile in peripheral blood $CD4^+$ T cells from patients with perennial allergic rhinitis and non-allergic rhinitis by FACS analysis.[11]

Pollinosis is thought to be an adaptive immune response that manifests as a type 1 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. Most effector T cells are short-lived, but few effector T cell become long-lived memory T cells. We directly examined the number of allergen-specific Th1/Th2 memory T cells in the peripheral blood of patients of allergic rhinitis by an ELISPOT assay using specific peptides.[12] The Japanese cedar-specific IL-4 producing Th2 cells were detected in all patients examined and 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 (Fig. 7) .

Allergen-specific immunotherapy is the only current treatment that can change the natural course of allergic rhinitis with long-term effects. However, the conventional immunotherapy with subcutaneous administration is inconvenient because it requires frequent visits to the doctor and also carries the risk of anaphylactic shock.[13] A recent review of randomized

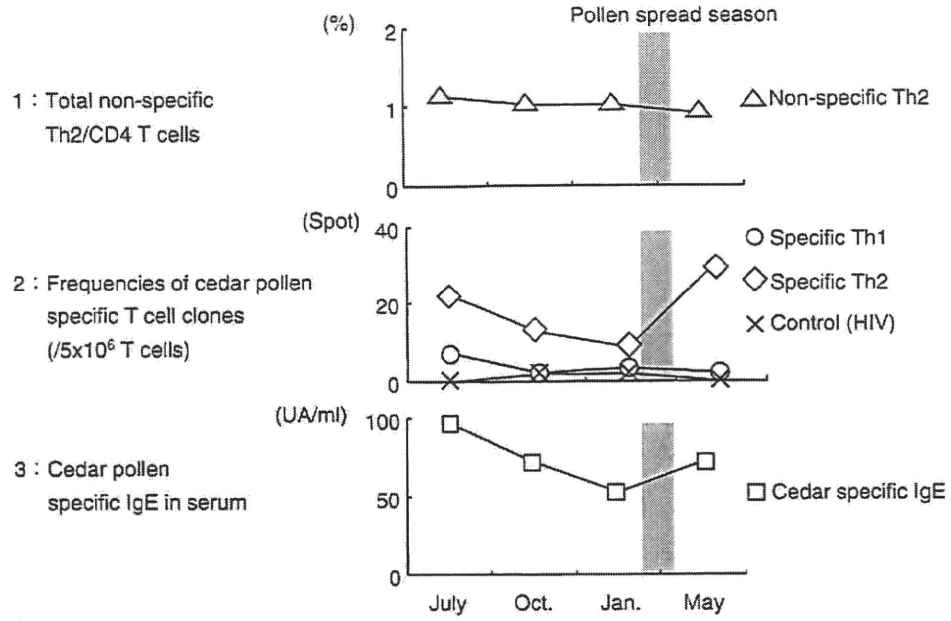

Fig. 7 The seasonal changes of total Th2 cells, frequency of cedar pollen specific T cell clones (spots number) and cedar pollen specific IgE.

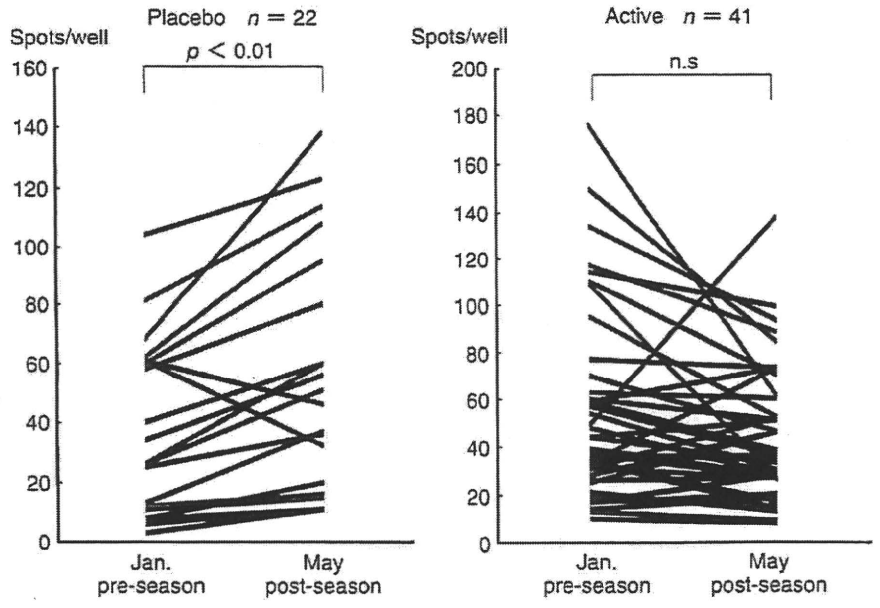

Fig. 8 The number of cedar-specific Th2 cells before and after sublingual immunotherapy.

controlled studies of sublingual immunotherapy suggested that this might be effective as an alternative method of administration.[14-16] To determine the efficacy of sublingual immunotherapy for Japanese cedar pollinosis, we conducted a blinded, randomized, placebo-controlled trial over a period of 6 months (from October 2005 to May 2006).[17] Sixty-seven subjects were enrolled and the nasal symptom scores during the cedar pollen season were evaluated using a symptom diary.

The patients in the active treatment group exhibited significantly lower symptom scores compared to the placebo group. This result suggests that sublingual immunotherapy may offer a safe approach to the management of allergic rhinitis, although the *in vivo* mechanisms of allergen-specific immunotherapy are

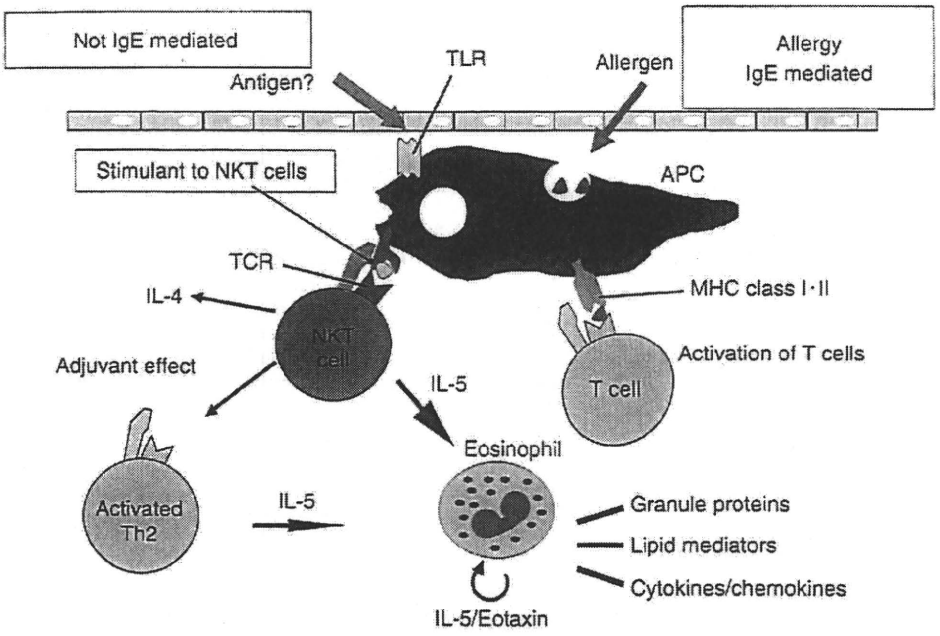

Fig. 9 Mechanism of eosinophil accumulation in respiratory mucosa. Eosinophil accumulation could be observed in MHC class-2 independent.

unknown.

Figure 8 shows the numbers of cedar-specific Th2 cells before and after immunotherapy: the number of Th2 memory cells increased in the placebo group after pollen exposure, but did not increase in the treatment group. Therefore, allergen-specific immunotherapy inhibits an increase in the antigen-specific Th2 memory cell count induced by allergen exposure. Immune-therapeutic intervention might direct at diminishing the size of the clone memory Th2 cells and shifting the cytokine type of memory Th clones.

Natural killer T (NKT) cells represent a unique lymphocyte subpopulation that is characterized by the co-expression of T cells and natural killer receptors.[18,19] Their activity is not restricted to MHC antigens. The relative frequency of NKT cells in the peripheral blood is generally quite low, usually less than 0.1% of PBMCs, and they are not detected in normal peripheral lymph nodes. However, NKT cells play a very important role in innate immunity. Recently, the involvement of NKT cells in the development of airway hypersensitivity in mice and the detection NKT cells in bronchoalveolar-lavage fluid samples from patients with moderate to severe asthma were reported. However, we could not detect the NKT cells in the nasal mucosa of the patients with allergic rhinitis by a polymerase chain reaction. However, NKT cells were detected to varying degrees in the sinus mucosa from asthmatic choronic sinusitis (CS) patients.

These results suggest that NKT cells are not directly related to the development of allergy, but that they may play important roles in the development of sinus disease combined with asthma and in the enhanced Th2 cytokine expression and increased infiltration of Th2 cells and eosinophils observed in the sinus mucosa from asthmatic CS patients via MHC-independent mechanisms (Fig. 9).

SUMMARY

1. The prevalence of allergic rhinitis, in particularly cedar pollinosis, is increasing.

2. Cedar pollen-specific Th1/Th2 dysregulation is observed in patients with pollinosis.

3. Cedar pollen specific memory Th cells increased during the pollen season and decreased during off season, however, morethan 60% of the memory cells survived up to 8 months after the pollen season.

4. NKT cells are not directly related to the development of allergic rhinitis, including pollinosis.

5. Different mechanisms in the accumulation of eosinophilia in the respiratory tract mucosa may exist.

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Reevaluation of pollen quantitation by an automatic pollen counter

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ABSTRACT

Accurate and detailed pollen monitoring is useful for selection of medication and for allergen avoidance in patients with allergic rhinitis. Burkard and Durham pollen samplers are commonly used, but are labor and time intensive. In contrast, automatic pollen counters allow simple real-time pollen counting; however, these instruments have difficulty in distinguishing pollen from small nonpollen airborne particles. Misidentification and underestimation rates for an automatic pollen counter were examined to improve the accuracy of the pollen count. The characteristics of the automatic pollen counter were determined in a chamber study with exposure to cedar pollens or soil grains. The cedar pollen counts were monitored in 2006 and 2007, and compared with those from a Durham sampler. The pollen counts from the automatic counter showed a good correlation ($r > 0.7$) with those from the Durham sampler when pollen dispersal was high, but a poor correlation ($r < 0.5$) when pollen dispersal was low. The new correction method, which took into account the misidentification and underestimation, improved this correlation to $r > 0.7$ during the pollen season. The accuracy of automatic pollen counting can be improved using a correction to include rates of underestimation and misidentification in a particular geographical area.

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In recent years, many countries have experienced an increase in the prevalence of pollinosis, as well as other allergic disorders.[1–3] The most important pollen allergens in Japan are tree pollens such as the Japanese cedar and Japanese cypress.[4–7] These correspond to grass pollens in European countries[8] and ragweed in the United States.[9] Monitoring of airborne pollens is useful because it allows selection of medication by physicians and allergen avoidance and self-care by patients through provision of pollen alerts based on specific pollen counts. The quantitation of pollen counts is commonly performed using the gravimetric Durham sampler or the volumetric Burkard sampler.[10,11] The Durham sampler is generally used in Japan, whereas the Burkard sampler is more common in Europe and America. However, these methods are time-consuming and difficult, resulting in poor performance in field settings and in other areas.

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These limitations have led to the development of automatic pollen monitoring using pollen counters that discriminate among pollens by size, shape, or self-fluorescence with a laser beam.[12–14] These instruments allow simple real-time automatic counting. However, distinguishing actual pollens from small nonpollen airborne particles remains a problem.[15] In particular, the cities of Tokyo and Chiba are located on the Kanto loam and have abundant loam grains. As reported previously,[15] the results from automatic pollen counters in Chiba show a good correlation with that from Burkard or Durham samplers at the peak of pollen scattering, with a relative good correlation coefficient, but a poor correlation in periods of low pollen scattering (<9 grains/cm^2 per day). This suggests that automatic pollen counters misidentify some soil grains as pollens. Therefore, in this study we developed a correction method using the rates of misidentification and underestimation of the automatic pollen counter to improve the accuracy of counting.

MATERIALS AND METHODS

Automatic Pollen Counter

The design of the automatic pollen counter (Shimyei Co., Ltd., Kobe, Japan) is based on that of a standard particle counter, in which a defined volume of air is circulated through a fine pipe that is intersected by a laser beam (Fig. 1). A scattered signal is detected when a particle passes through the laser beam. The intensity of this signal is related to the particle size and optical

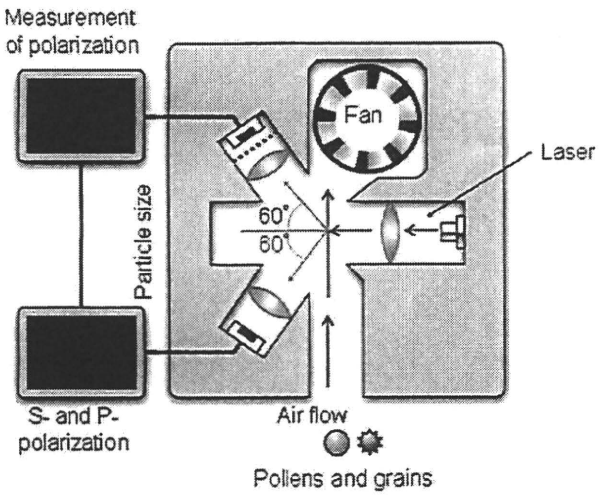

Figure 1. Optical configuration of the Shinyei automatic pollen counter. The counter distinguishes pollens from soils by size and polarization.

index, allowing the intensity of the scattered light to be related to the particle size. In addition to the scattering intensity, the automatic counter includes measurement of the change in the polarization state of scattered light, which is related to the shape and internal structure of the particle. Pollen grains give intensity and photopolarization signals that differ from those of nonpollen particles, which allows pollen to be recognized from two measurements. Identification of the pollen is based on a particle size of 10–30 μm and the polarization signal (Fig. 2) using the software of the automatic pollen counter, and >90% of the particles recognized as pollens by this automatic counter are thought to be actual pollen particles.

Chamber Study

The automatic pollen counter was placed in a 1-m^3 chamber into which cedar pollen (1499 grains; purchased from SRL Co., Tokyo, Japan) or Kanto loam grains (2488 grains) screened through a 100-μm filter were scattered. The number of small particles and pollens recognized by the counter were compared.

Underestimation and Misidentification Rates of the Automatic Pollen Counter

The pollen count (γ) from the automatic counter is given by $\gamma = \alpha \times (Z - Y) + (\beta \times Y)$, where X is the count of pollen particles, Y is the count of soil particles, Z is the count of all particles ($Z = X + Y$), α is the pollen identification rate by the counter, and β is the misidentification rate of soil particles as pollens. A new correction equation was developed as $X = \beta/(\beta - \alpha) \times Z - 1/(\beta - \alpha) \times \gamma$, where $\beta/(\beta - \alpha)$ and $1/(\beta - \alpha)$ are the pollen underestimation and misidentification rates, respectively. The value of α was fixed at 0.501 by the software of the counter.

A Correction Method Based on the Pollen Misidentification Rate in a Particular Area

Durham pollen samplers and automatic pollen counters were installed in the cities of Chiba, Narita, and Kobe. The pollen season usually lasts from the beginning of February to the end of April in these areas. The pollen misidentification rate was examined in each area and year from January 1 to 31 (just before the pollen season), and counts of soil particles misidentified as pollens were divided by the count for all particles in January (β). The number of pollens recognized by the automatic pollen counter and the revised number obtained by the new correction formula using the underestimation and misidentification rates were examined in the cedar pollen season.

Statistical Analysis

Correlation of data from the two pollen counters was examined based on daily pollen counts. Output for pollen collection in real time for the automatic pollen counter data was accumulated over 1 day based on hourly averages. The correlation coefficient (r) was calculated between the accumulated average m_x and the automatic pollen counter average m_y over n data points, with $r > 0.4$ and $r > 0.7$ taken to indicate low and good correlations, respectively.

RESULTS

Identification of Pollen and Soil Particles by the Automatic Pollen Counter in the Chamber

In the chamber study, only the pollens or the soil grains were scattered and the all of counts by the automatic counters meant the actual number of the pollens or the soils. The size and polarization of cedar pollens scattered in the chamber were calculated to be 20–40 μm and −0.4–0.8, respectively, by the automatic pollen counter (Fig. 3). Some pollens showed lower polarization and were identified as broken pollen by microscopic examination (data not shown). Similarly, the size and polarization of scattered soil particles (Kanto loam) were calculated to be 10–45 μm and −0.4–1.0, respectively. Identification of pollen by the recognition software of the automatic counter included the particles enclosed by the yellow line in Fig. 3. Some pollens were missed and a significant number of soil particles were recognized incorrectly as pollen particles.

Misidentification Rate of Soil Particles as Pollen Particles

The rate at which the automatic pollen counter misidentified soil grains as pollens (Fig. 4) was cal-

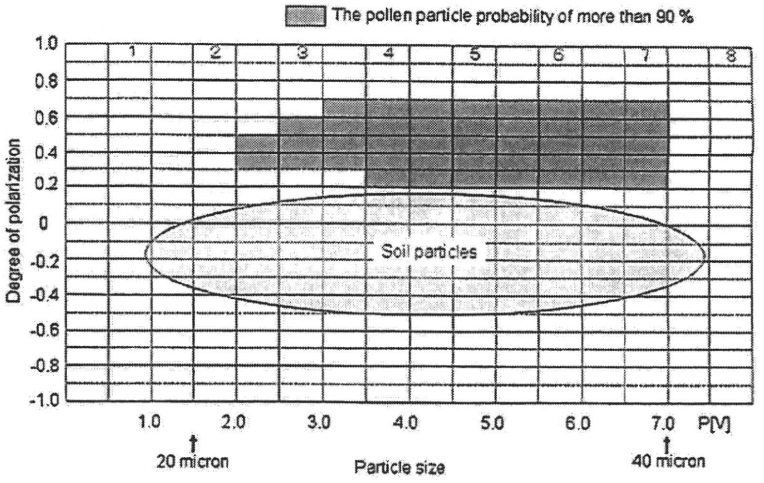

Figure 2. Original method of pollen particle identification built in the Shinyei automatic pollen counter. The horizontal bar indicates the size that was originally expressed by the electric resistance P[V] and is logarithmic correlation with particle size (micron). The vertical bar shows the polarization. Identification of the pollen by the automatic counter is based on a particle size of 10–30 μm and the polarization signal and >90% of the particles recognized as pollens by this automatic counter are thought to be actual pollen particles.

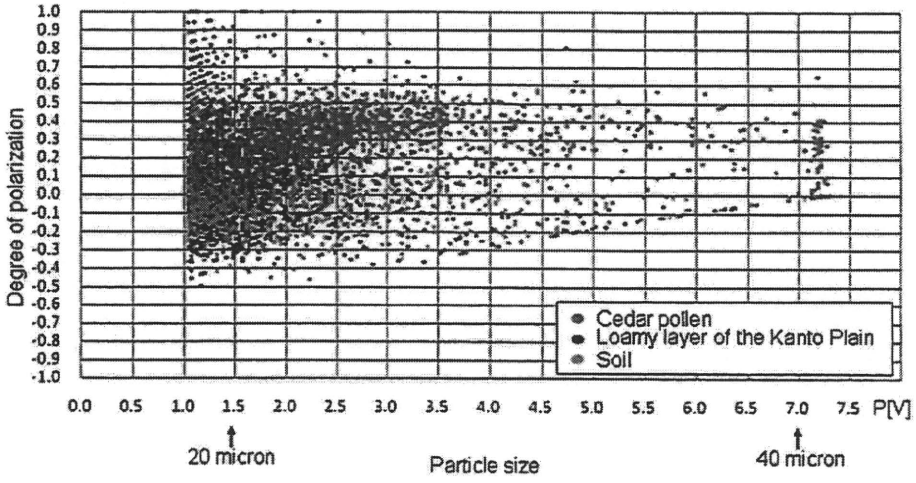

Figure 3. Counts of pollens and soil particles in the chamber study. Some pollens were underestimated and some soils were misidentified as pollens.

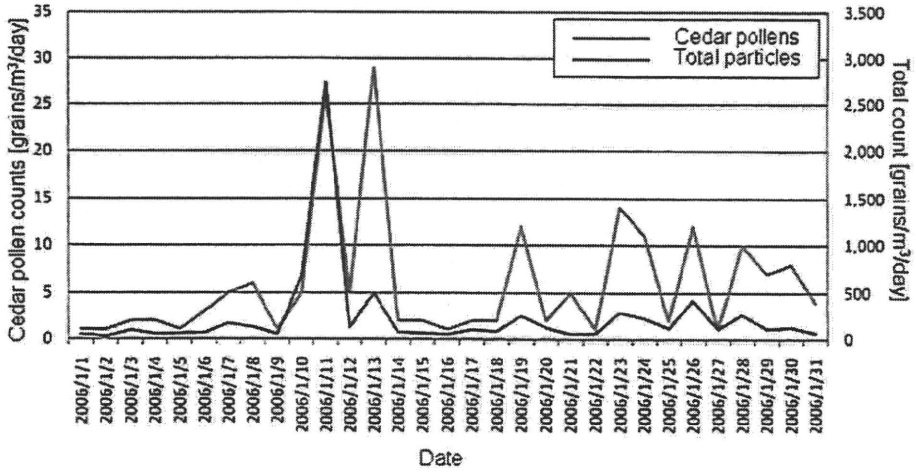

Figure 4. Misidentification of soil particles as pollens and all particles recognized by the automatic pollen counter in January 2006 (out of pollen season) in Narita City. Average misidentification rate of soils as pollens was calculated as 0.034.

culated from the average pollen misidentification count and the total count of all particles in January 2006, 2007, and 2008 (out of the pollen season). The rates in these years were 0.068, 0.065, and 0.111, respectively, in Chiba; and 0.073, 0.064, and 0.110, respectively, in Narita.

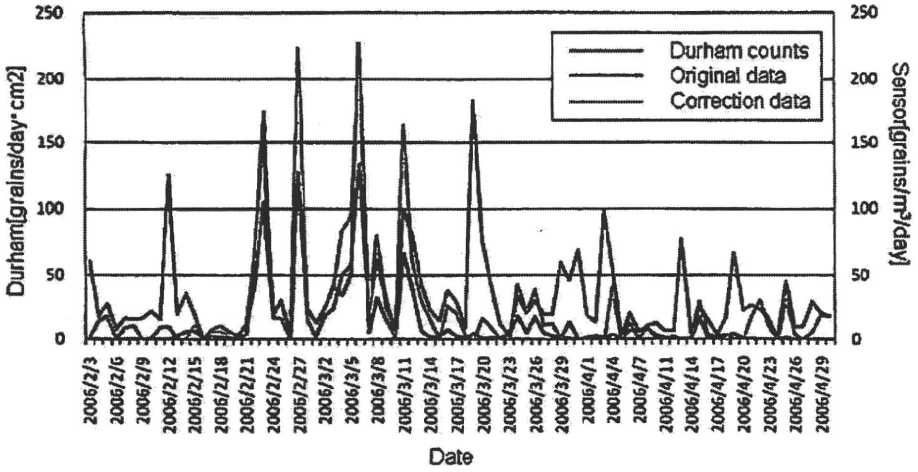

Figure 5. Daily pollen count from the Durham sampler and from the automatic pollen counter with and without correction from February 3 to April 30 in 2006 in Narita City. Correlation rate of pollen counts by the automatic counter with those by Durham sampler was 0.54 without correction and 0.91 with correction in Narita City.

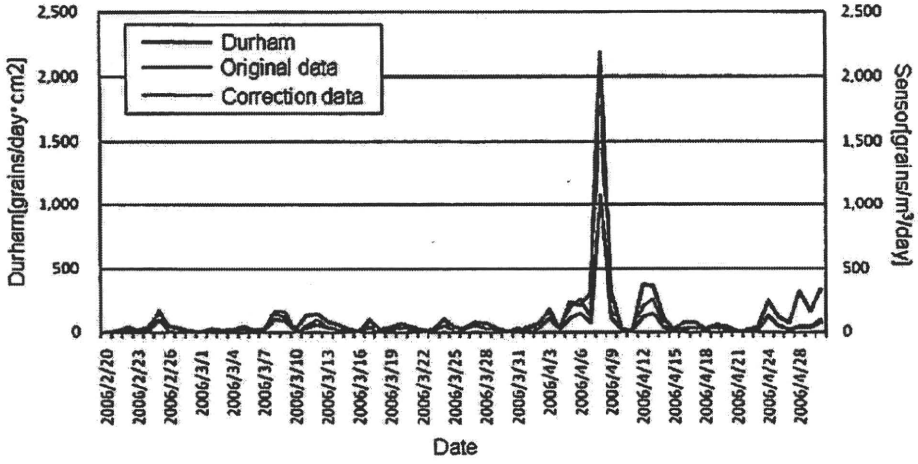

Figure 6. Daily pollen count from the Durham sampler and from the automatic pollen counter with and without correction from February 3 to April 30 in 2006 in Kobe City. Correlation rate of pollen counts by the automatic counter with those by Durham sampler was 0.98 without correction and 0.98 with correction in Kobe City.

The Effect of Correction Using a New Method

The daily cedar and cypress counts in the pollen season obtained with the Durham sampler and the automatic counter in Narita and Kobe are shown in Figs. 5 and 6, respectively. Without correction, the pollen count from the automatic counter showed a poor correlation with that from the Durham sampler in Narita. This correlation was improved by correction using the new method. In Kobe, the correlation was good with or without correction. The correlation rates between the daily pollen counts from the automatic pollen counter and the Durham sampler over the whole pollen season in Chiba and Narita were summarized in Table 1. The correlation in Kobe was high and there was no improvement after correction. In contrast, in the other cities the uncorrected correlation rate was low in 2006 and 2007 and was improved ($r > 0.7$) by correction with the new method. When the original counts showed good correlations with Durham counts, the new method did not improve more.

Table 1 **Correlation of pollen counts from the automatic pollen counter with those from the Durham pollen sampler**

	Year	Without Correction	With Correction
Chiba	2006	0.43	0.79
Narita	2006	0.54	0.91
Chiba	2007	0.50	0.77
Narita	2007	0.66	0.80
Chiba	2008	0.80	0.76
Narita	2008	0.78	0.87

DISCUSSION

The particle size and photopolarization of pollens are nonuniform and can be diverse. The particle size of loam grains shows significant overlap with that of cedar pollen, but the low polarization of loam grains improves discrimination from cedar pollen by the automatic pollen counter. However, some soil particles

show similar polarization to cedar pollen. In particular, the cities of Chiba and Narita are covered with Kanto loam, and monsoons in these areas during the pollen season cause increases in airborne particles and misidentification rates. In contrast, Kobe is not covered with loam and faces the sea, which decreases the number of airborne particles in Kobe.

Pollen dispersal information using automatic pollen counters is provided by the Ministry of the Environment of Japan, local government, and some companies.[10] However, patients with pollinosis often feel that this information differs from the actual conditions and self-care may be hindered by incorrect information because of misidentification and overestimation by automatic pollen counters.

In counting of pollens, the Shinyei automatic pollen counter excludes particles of similar size or polarization to soil particles.[14] The pollens detected by the Shinyei counter include >90% of actual pollens and <10% of soil grains.[14] However, the counter still has difficulty in discriminating pollens from soil grains, particularly in periods when scattering of pollen is low and that of soil grains is high.[15] The final pollen count (X) is calculated as $X = (\beta/(\beta - \alpha) \times Z - 1/(\beta - \alpha) \times \gamma$, as described in the Methods section, where α, the pollen identification rate, is set at 0.501 in the counter, β is the erroneous recognition rate of soil particles as pollens, and γ is the total count of particles identified as pollens by the counter. $\beta/(\beta - \alpha)$ and $1/(\beta - \alpha)$ are defined as the pollen underestimation and misidentification rates, respectively. In this formula, β is a variable and the levels and properties of soil grains influence the value of β.

We calculated the misidentification rate of the automatic pollen counter in each area in January, just before the start of the pollen dispersal season but when the weather is similar to that during the pollen season. Subsequent pollen counts were then revised according to the new correction method using the misidentification and underestimation rates. This correction was found to improve the accuracy of the automatic pollen counts compared with those from the Durham pollen sampler when the original count showed a poor correlation with the Durham count. Comparison with Burkard counts obtained using the volumetric method was not performed because use of Burkard samplers is very limited in Japan, whereas Durham samplers are widely used. This is a limitation of the study. The new correction method did not improve the correlation when the original count showed a good correlation with the Durham count. The annual pollen counts (per cm^2) by Durham sampler in 2006, 2007, and 2008 were 1155, 2777, and 6596, respectively, in Chiba and 1162, 2642, and 5685, respectively, in Narita. For the last 10 years, the average pollen count by Durham sampler was 3300 grains/cm^2 per season in Chiba. In the classification of the pollen count by Durham sampler, 0–9 grains/cm^2 per day is defined as low pollen scattering.[10] As reported previously, the automatic pollen counters showed poor correlation with the Durham sampler in the period of low pollen scattering. The new correction method may be useful in a season of low pollen scattering in an area rich in loam grains or other nonpollen airborne particles.

Precise monitoring of airborne pollens is useful to both doctors and patients for allergen avoidance and self-care.[1,10] Automatic pollen counters discriminate pollens based on size and polarization by a laser beam, and can allow simple real-time automatic counting. Exact distinction from soil grains is difficult because pollens are diverse and their size and polarization overlap with some nonpollen particles, but this limitation is reduced by the correction proposed in this study. We examined the misidentification and underestimation rates of the automatic pollen counter to improve the accuracy of the counting. The misidentification rate can be calculated just before pollen scattering yearly in each area, and correction using this rate is likely to improve the accuracy of pollen monitoring. The size and polarity of cedar pollen are similar to those of orchard pollen and timothy pollen, but significantly different from those of birch pollen and ragweed pollen (data not shown). Therefore, discrimination between cedar pollen and birch or ragweed pollen might be possible.

Detection of the start of pollen scattering is difficult and it may still be necessary to use the automatic counter together with a conventional method such as a Durham sampler; however, maintenance of the automatic counter is easy and wide distribution of these counters should enable general use and permit real-time pollen scattering information to be obtained.

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分担研究報告書

アルタイムモニター花粉数の情報のあり方の研究と舌下ペプチド・アジュバント療法の臨床研究

花粉症の地域特異性と花粉抗原の共通抗原性および重症スギ花粉症患者の局所病態

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研究要旨

スギ花粉症は、通年性アレルギー性鼻炎とは異なり、特定の時期に大量の花粉が飛散することによって非常に強い局所症状のみならず全身症状を引き起こす。近年スギ花粉症の患者数は急増し、国民の約20%を超えると報告されており、現代社会においてこの疾患の治療は重要性を増している。スギ花粉症に罹患する患者層は若年層が多く、治療はくしゃみ、鼻汁、鼻閉などの鼻、眼の症状だけではなく、倦怠、集中力の低下など日中のパフォーマンスが労働や就学に及ぼす影響があるためにQOLを考慮した治療が求められている。この治療にあたってはスギ花粉飛散数の把握が極めて重要であり、重症のスギ花粉症患者の局所での病態を解明することが新しい治療方法の展開に必須である。また、免疫療法で使用する抗原を選択するためには、花粉抗原の特性特に共通抗原性に関する検討が極めて重要である。そこで今回、われわれは山形市における花粉症の特異性を検討するために、種々の花粉抗原陽性率を検討するとともにこれら花粉抗原の共通抗原性についても検討した。さらに、スギ花粉飛散数がスギ花粉症患者の症状、QOLおよび睡眠障害に及ぼす影響について検討した。また、重症スギ花粉症患者の鼻粘膜におけるステロイド受容体、ペンドリン、ペリオスチンなどの分子の発現について検討を加えた。その結果、山形市ではスギ、イネ科だけでなくコナラ、ヤナギ、クルミ、シラカバ、ヒメシイバによる感作が確認され、コナラ、ヤナギ、クルミ、シラカバ、ヒメシイバにはそれぞれ共通抗原性が認められた。また、スギ花粉飛散数の増加に伴い、鼻眼の症状が増悪するだけでなく、QOLも悪化し、睡眠も障害されることが示唆された。このような重症のスギ花粉症患者の鼻粘膜にはステロイドβ受容体、ペリオスチン、ペンドリンが過剰に発現しており、このためステロイド抵抗性、リモデリングおよび鼻汁の過剰分泌が生じ、これらの変化が重症化の一因と考えられた。

A. 研究目的

スギ花粉症は、通年性アレルギー性鼻炎とは異なり、特定の時期に大量の花粉が飛散することによって非常に強い局所症状のみならず全身症状を引き起こす。近年スギ花粉症の患者数は急増し、国民の約20%を超えると報告されており、現代社会においてこの疾患の治療は重要性を増している。スギ花粉症に罹患する患者層は若年層が多く、治療はくしゃみ、鼻汁、鼻閉などの鼻、眼の症状だけではなく、倦怠、集中力の低下など日中のパフォーマンスが労働や就学に及ぼす影響があるためにQOLを考慮した治療が求められている。この治療にあたってはスギ花粉飛散数の把握が極めて重要であり、重症のスギ花粉症患者の局所での病態を解明することが新しい治療方法の展開に必須である。また、免疫療法で使用する抗原を選択するためには、花粉抗原の特性特に共通抗原性に関する検討が極めて重要である。そこで今回、われわれは山形市、甲府市、福井市の3市のスギ花粉飛散数について検討した。また、重症スギ花粉症患者の鼻粘膜におけるステロイド受容体、ペンドリン、ペリオスチンなどの分子の発現について検討した。さらに、スギ、イネ科花粉、コナラ、ヤナギ、クルミ、シラカバ等の花粉抗原の共通性について検討した。

B. 研究方法

スギ花粉飛散数の測定：スギ花粉飛散数は、リアルタイムモニター(KH3000)を用いて山形市(山形大学医学部および山形県衛生研究所の屋上)、福井市(福井大学および福井県大気汚染測定局の屋上)、および甲府市(山梨大学および山梨県衛生研究所の屋上)にて測定した。

QOLおよび睡眠障害の検討：奥田らの作成したJRQLQ(No1およびNo2)調査票を用いた。JRQLQの評価はそれぞれの重症度を0から4の5段階でスコア化し各々の障害の程度を分析、さらに総合スコアを求めて評価を行った。また、睡眠障害についてはピッツバーグ睡眠問診票(PSQI)を用いた。評価方法であるが、睡眠時間以外は重症度を

0から3の4段階で評価し、さらに通常総合スコアを用いて睡眠障害の程度について評価した。
ステロイド受容体、ペリオスチンおよびペンドリンの測定：重症スギ花粉症患者の鼻粘膜におけるステロイド受容体（αおよびβ受容体)、ペリオスチンおよびペンドリンの発現を免疫組織学的に検討した。ペンドリンおよびペリオスチンの発現は、Real Time PCR および Western blotting を用いた検討も行った。
RAST インヒビション：ヒメシイバ、コナラ、ヤナギ、クルミ、シラカバ等の花粉抗原の共通性について、患者血清と花粉抗原エキスをそれぞれ inhibitory antigen として反応させた後、HRP 標識抗ヒト IgE 抗体を加えさらに 1 時間室温にて反応させた．その後発色液を加え ELISA にてプレートに固相化された抗原と反応した血清 IgE の量を定量し評価した．
｛(positive control の吸光度）－（それぞれの吸光度)｝/（positive control の吸光度）×100%の式でプレートへの IgE 吸着率を計算し，高濃度でプレートと同一抗原にて反応させた血清の IgE 量おける値を 100%に換算し評価した．

C. 結果
スギ花粉飛散ピーク時の JRQLQ(No2)の全項目について、健常人とスギ花粉症患者のスコアを比較検討すると、眼鼻の症状が増悪するだけでなく、気道、のど、口耳皮膚、全身に影響が及ぶことが確認された。 健常人とスギ花粉症患者の睡眠障害について PSQI を用いて比較検討した。花粉飛散初期には、健常人とスギ花粉症患者で明らかな差は認められなかった。しかし、花粉飛散ピーク時期では、健常人は睡眠スコアの有意な上昇は認められなかったのに対して、スギ花粉症患者では有意な上昇が認められ、睡眠が障害されている可能性が示唆された。重症アレルギー性鼻炎患者の鼻粘膜では、グルココルチコイドβ受容体の発現が増強しており、ステロイド耐性状態であることが示唆された。また、ペリオスチンは上皮の基底膜に強く発現し、ペンドリンは腺細胞で陽性であった。これらの所見から、気管支喘息の粘液分泌過多とリモデリングに相当する変化が鼻粘膜でも生じていると考えられた。さらに、花粉抗原の共通抗原性について検討した。その結果、シラカンバ抗原に対してはコナラ・ヤナギ抗原、クルミ抗原に対してはシラカンバ・コナラ・ヤナギ抗原、ヒメスイバ抗原に対してはシラカンバ抗原、コナラ抗原に対してはクルミ抗原、ヤナギ抗原に対してはシラカンバ・コナラ抗原において添加した抗原の濃度に依存した抑制が掛かり、何らかの共通抗原性を疑う結果を得た。

D. 考察：
山形市、福井市、甲府市の 3 地域での複数の施設でのスギ花粉飛散数は、花粉開始時期、最大飛散数、日々の飛散数の変化はほぼ一致していたが、部分的に異なる傾向もありよりきめの細かい花粉飛散状況の情報提供には複数施設の利点を生かすことも一案と考えられた。また、アレルギー炎症の重症化の機序には、糖質コルチコイド受容体、ペリオスチン、ペンドリンなどの分子が関与している可能性が示唆された。特に、グルココルチコイドのαβ受容体の発現のバランスが重要であり、気管支喘息や潰瘍性大腸炎などでβ受容体の発現が亢進している症例では、ステロイドの抵抗性が高いことが報告されている。今回の検討でも、ステロイドに抵抗する症例の局所ではβ受容体の発現が高い傾向が認められた。また、花粉抗原の共通抗原性について検討したところ、ヒメシイバ、コナラ、ヤナギ、クルミ、シラカバに共通抗原性がある可能性が示唆された。

E. 結論
スギ花粉飛散数は同一市内の複数地点での測定ではその変動と数はほぼ一致した。重症アレルギー性鼻炎症例の鼻粘膜では、グルココルチコイドβ受容体、ペンドリン、ペリオスチンなどの発現が亢進しており、これらの変化が重症化の一因と考えられた。また、種々の花粉抗原は共通抗原性を有しており、花粉飛散が重なる初夏にはこの点を念頭に置き治療戦略を立てる必要があると考えられた。

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G.　知的所有権の取得状況

１．特許取得　なし
２．実用新案登録　なし
３．その他

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ORIGINAL PAPER

Development of electron spin resonance radical immunoassay for measurement of airborne orchard grass (*Dactylis glomerata*) pollen antigens

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Abstract We have developed a highly sensitive method for the measurement of airborne orchard grass (*Dactylis glomerata*: Dac g) pollen antigens using an electron spin resonance (ESR) radical immunoassay. In this immunoassay, the lowest detectable level of Dac g antigen in a sample is 0.1 arbitrary unit; the amount of Dac g antigen in single pollen grains was found to be as 1.84 units. Thus, Dac g antigens can be detected in amounts of 1/20th of that contained in the grain. This immunoassay enables early detection of grass pollen antigens. Such information may be useful for patients with grass pollinosis, especially for those who show symptoms when only low levels of the pollen antigens are present in air. In this study, minor amounts of Dac g antigen (cross-reactive antigens) were detected in late March, after which the levels gradually increased. The levels were detected to be 10 units/m^3 until the middle of May and then increased after blooming of orchard grass. High levels were maintained until the middle of June. Some patients who suffer from grass pollinosis show symptoms in late April and early May, when the airborne Dac g antigen levels were found to be 5–10 units/m^3.

Keywords Airborne pollen · Allergen · ESR radical immunoassay · Orchard grass · Japanese cedar

Abbreviations

BCA	Bicinchoninic acid
BCIP/NBT	5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium
BSA	Bovine serum albumin
CNBr	Cyanogen bromide
Dac g	*Dactylis glomerata*
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal bovine serum
ESR	Electron spin resonance
HRP	Horseradish peroxidase
MONALISA	Monitoring Network of Allergen by Immuno-Sampling

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