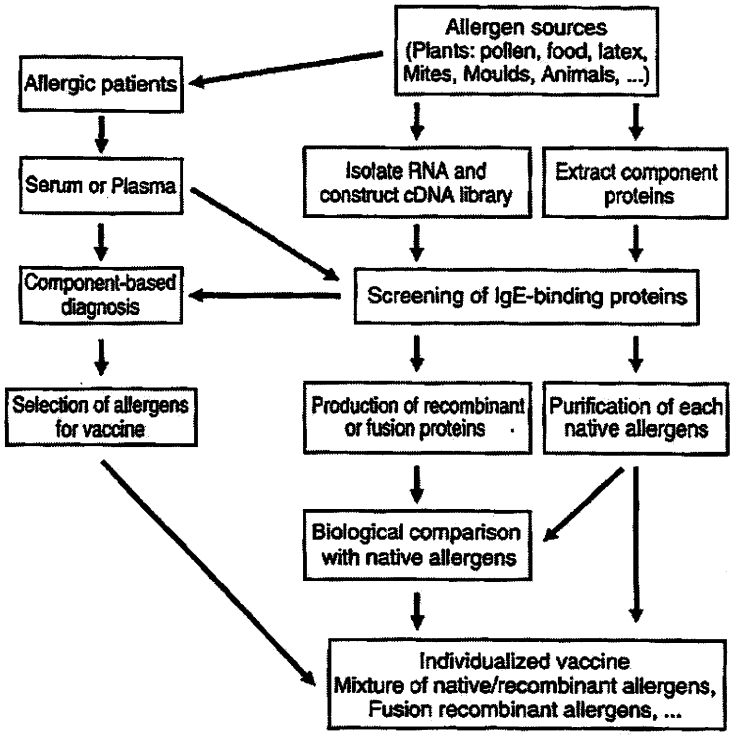

Fig. 1 Schematic procedure of the steps involved in the identification and development of an individualized vaccine using only sensitized antigens for immunotherapy. To identify component allergens which have the capacity to react with serum IgE from allergic patients, it is important to establish individualized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

ROUTE OF VACCINE ADMINISTRATION FOR IMMUNOTHERAPY AND ITS SAFETY

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

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

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

Table 1 Comparison between SLIT and SCIT

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

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

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

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

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

BIOMARKERS FOR SLIT

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

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

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

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

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

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

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

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

MECHANISMS OF ANTIGEN-SPECIFIC IMMUNOTHERAPY

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

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

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

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

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

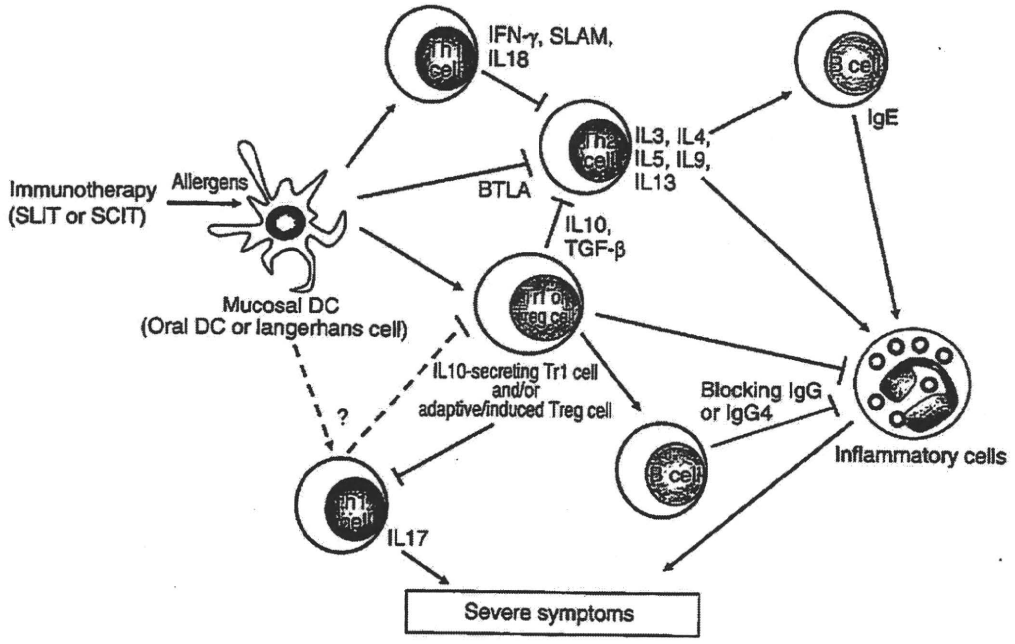

Fig. 2 T cells in antigen-specific immunotherapy. Antigen-specific immunotherapy induces regulatory T cells and Th1 cells via antigen-presentation by mucosal dendritic cells (DC). Th17 cells may be induced in a non-responder population by immunotherapy. The induced Th1 cells and/or regulatory T cells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

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

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

ANTIGEN-SPECIFIC IMMUNOTHERAPY AGAINST JAPANESE CEDAR POLLINOSIS

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

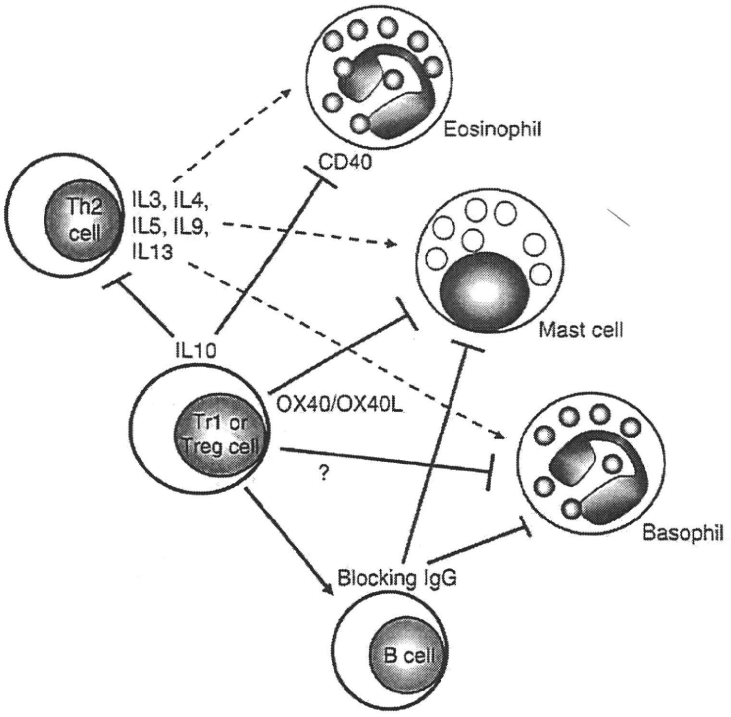

Fig. 3 Proposed roles of regulatory T cells on inflammatory cells in allergen-specific immunotherapy. Regulatory T cells, namely IL10-secreting Tr1 cells or adaptive/induced Treg cells, down-regulate inflammatory cells, directly or indirectly. Regulatory T cells down-regulate the activation of Th2 cells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

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

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

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

tained Cry j 1-Cry j 2 fusion protein in the immunoregulatory liposome showed suppression of IgE and IgG antibody responses after being challenged with the allergens. Furthermore, oral administration of the vaccine showed efficient suppression of IgE antibody production.[74]

CONCLUSIONS

The standardization of a vaccine enables us to compare the results from varied clinical trials with respect to dose, clinical effects, and changes in biological parameters. Many reports have shown positive clinical therapeutic effects and suppressed effector/inflammatory responses. It is considered that IL10-producing Tr1 and/or adaptive or induced Treg cells may be involved in the suppression of the antigen-specific Th2-responses and local inflammation. However, how immunotherapy induces suppressor cells like Tr1 and Treg cells remains unclear, although the involvement of mucosal dendritic cells has been proposed. High-quality clinical studies are indispensable to clarify the therapeutic biomarkers and the mechanisms of induction of suppressor cells, and the resultant data from the studies may enable us to develop safer and more effective immunotherapy through the modification of the allergens, optimum dose, or administration regimen of a vaccine.

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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

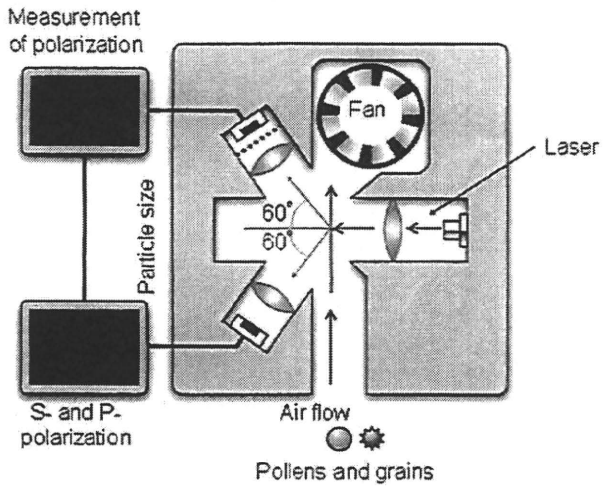

Figure 1. Optical configuration of the Shinyei automatic pollen counter. The counter distinguishes pollens from soils by size and polarization.

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-

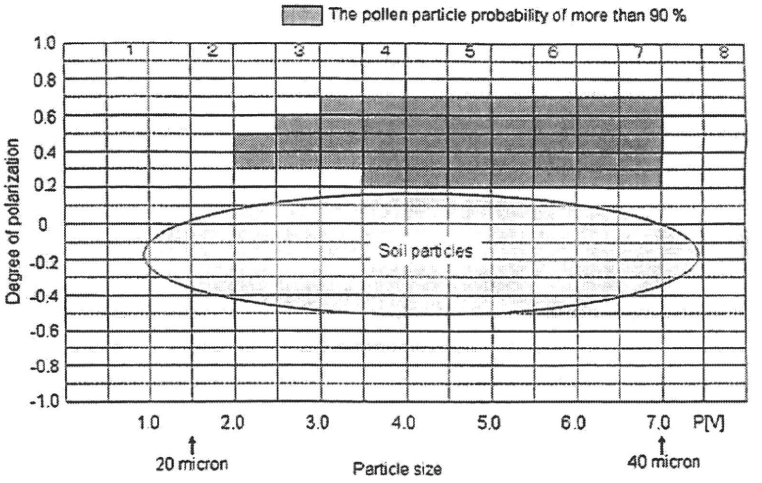

Figure 2. Original method of pollen particle identification built in the Shinyei automatic pollen counter. The horizontal bar indicates the size that was originally expressed by the electric resistance P[V] and is logarithmic correlation with particle size (micron). The vertical bar shows the polarization. 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.

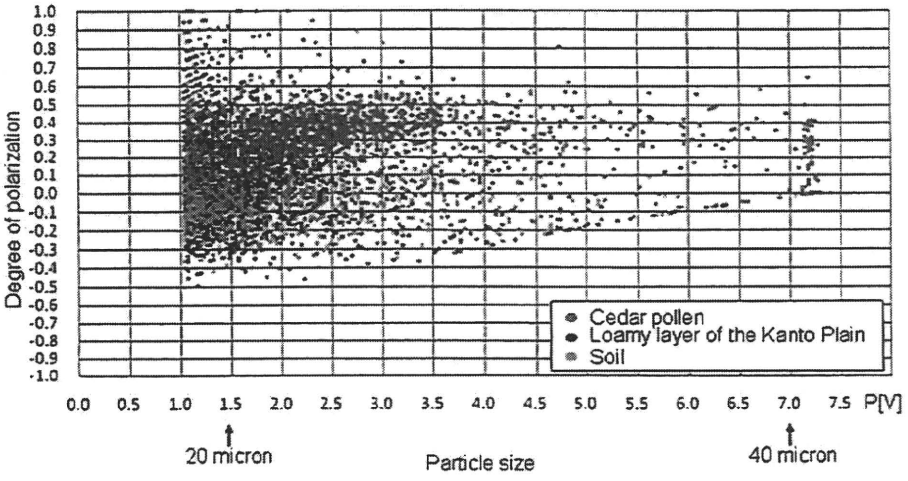

Figure 3. Counts of pollens and soil particles in the chamber study. Some pollens were underestimated and some soils were misidentified as pollens.

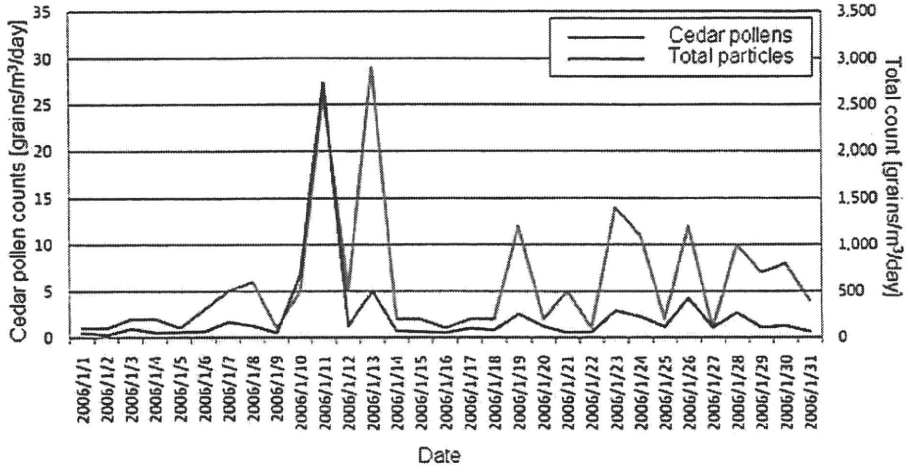

Figure 4. Misidentification of soil particles as pollens and all particles recognized by the automatic pollen counter in January 2006 (out of pollen season) in Narita City. Average misidentification rate of soils as pollens was calculated as 0.034.

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.

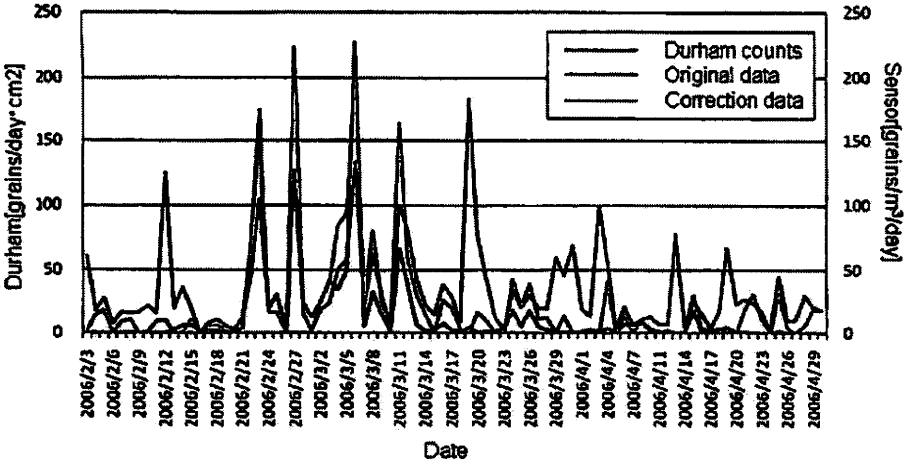

Figure 5. Daily pollen count from the Durham sampler and from the automatic pollen counter with and without correction from February 3 to April 30 in 2006 in Narita City. Correlation rate of pollen counts by the automatic counter with those by Durham sampler was 0.54 without correction and 0.91 with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

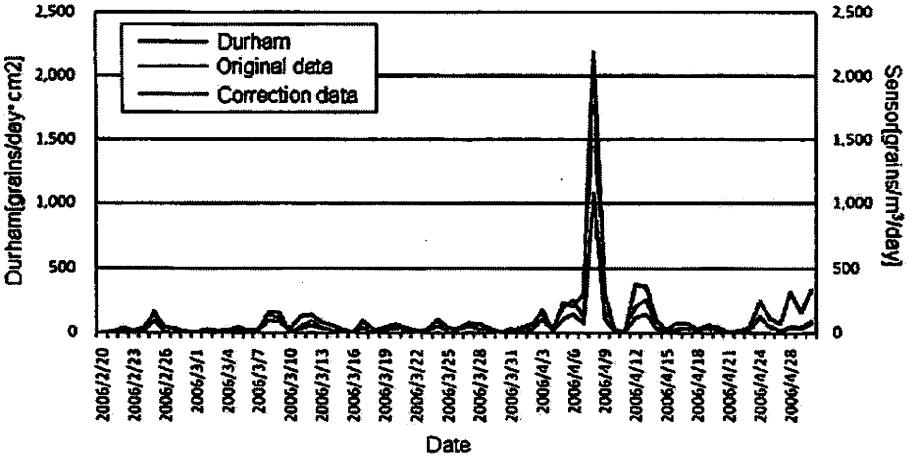

Figure 6. Daily pollen count from the Durham sampler and from the automatic pollen counter with and without correction from February 3 to April 30 in 2006 in Kobe City. Correlation rate of pollen counts by the automatic counter with those by Durham sampler was 0.98 without correction and 0.98 with correction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

スギ花粉症患者における呼気中一酸化窒素濃度に関する研究

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研究要旨
季節性の鼻炎症状を呈するスギ花粉症患者に対して、スギ花粉飛散前と飛散時期に気管支喘息における下気道の炎症マーカーである呼気 NO 濃度を検討した。これらの症例において、呼気 NO 濃度は飛散前期と比較して飛散期においては有意に上昇していた($p < 0.05$)。呼吸機能検査では、スギ花粉飛散時期の前後において、VC，%VC，FEV1%，及びピークフロー（PEF）は有意な変動は認められなかった。スギ花粉症では鼻結膜炎症状のみならず、下気道においてもサブクリニカルな炎症病態が発現していることが示唆された。

A. 研究目的

スギ花粉症では一般に気管支喘息症状を呈する症例はまれとされてきた。一方で基礎疾患として気管支喘息がある場合にはその限りでなく、我々のものを含む複数の報告が、スギ飛散時期に合致して既存の喘息の増悪がみられることが報告されてきた。

同一アレルゲンが鼻炎症状とときに喘息症状の両疾患をもたらすといういわゆる“one airway one disease”の概念のもとに、花粉症から喘息の移行予防に関する研究はいろいろ行われているが、早期発見での視点の報告は少ない。

われわれは、臨床的な気管支喘息の合併がないスギ花粉症症例を対象に、携帯用 NO 測定機器 NIOX MINO（Aerocrine 社）を用い、気管支喘息における下気道のアレルギー性炎症を評価する新規バイオマーカー検査である呼気NO濃度についての検討を行った。

B. 研究方法

通年症状がなく、純粋に２～４月のスギ花粉飛散時期のみに鼻炎症状を呈する、スギ花粉症症例 7 例を対象とした。全例が非喘息症例であり、非喫煙者であった。慢性閉塞性肺疾患(COPD)、気管支拡張症、びまん性汎細気管支炎などの慢性の気道性呼吸器疾患、心不全、脳血管障害など重症の基礎疾患を有する症例、妊娠している症例、鼻副鼻腔手術既往症例、う歯、歯科治療中の症例は除外した。

スギ花粉飛散前と飛散時期に、症状を評価し、呼気 NO ならびに呼吸機能検査を施行した。鼻症状の重症度評価は、鼻アレルギー診療ガイドラインに準拠しておこなった。呼吸機能検査は、オートスパイロ 307(MINATO 社製)を使用して行った。呼気 NO 濃度は、携帯用 NO 測定機器 NIOX MINO（Aerocrine 社）で測定した。

C. 研究結果

今回エントリイしたスギ花粉症症例において、スギ花粉飛散前期にあらかじめ呼気 NO が 40ppb 以上（喘息と診断しえるレベルの異常高値）であった症例が 1 症例存在した。これを除外した、６症例について主に季節性のアレルゲン暴露に伴う変化の追跡を行った。

これらの６症例において、各症状、鼻内所見は、鼻腔容積変化を除くすべてにおいて、スギ花粉飛散期に症状は悪化し、鼻内所見でも有意な変化を示した($p < 0.05$)。

呼気 NO 濃度については、飛散期、非飛散期で有意差を認め、飛散期で上昇していた($p < 0.05$)。

各症状、鼻内所見と NO との相関性では、花粉症症例、非花粉症症例ともに有意な相関性は認められなかった。

呼吸機能検査では、スギ花粉飛散時期の前後において、VC，%VC，FEV1%，及びピークフロー（PEF）は有意な変動は認められなかった。なおこれらの６症例においては、スギ花粉飛散時期においても、明らかな気管支喘息症状を呈した症例はみられなかった。

D. 考察：

今回検討したスギ花粉症症例においては、呼気

NOが、飛散期と非飛散期とで有意差が認められ、飛散時期における明らかな上昇が認められた。このことから、スギ花粉症症例では、気管支喘息に代表される下気道のアレルギー症状を一見呈していないケースにおいても、潜在的には下気道にアレルギー性炎症を生じていることが示唆された。
これらの症例では喘息症状自体の発現、あるいは呼吸機能検査での気流制限、閉塞性換気障害の発現などはみられておらず、純粋なスギ花粉症患者においてのみみると、かかる下気道での炎症病態は臨床的な喘息を発症する前段階的なものであることが推察された。
鼻炎症状、鼻内所見については鼻腔容積の変化を除き、各症状、鼻内所見は、花粉飛散期と非飛散期で有意差を認めたが、呼気NOとの有意な相関性は認めなかった。
呼吸機能検査についても、スギ花粉飛散前と飛散中で、VC, %VC, FEV1%, 及びピークフロー(PEF)は、有意な変動は見られなかった。
症例数が少ないため、スギ花粉症による呼吸機能への影響の頻度、大きさまでは言及できないが、呼気NOは、呼吸機能とは相間性を認めず、しかしながらスギ花粉飛散により有意な上昇が認められたことから、呼吸機能検査よりも早期に下気道の病態の変化を察知している可能性があると考えられる。

E. 結論

スギ花粉症の症例では喘息症状に代表される下気道の症状を訴えていない段階で、すでに気管支喘息様のアレルギー性炎症が下気道に発現している症例が存在する。
かかる成績が示唆するものは、鼻のみに対する例えば点鼻ステロイド療法であるとか、あるいはレーザー治療などといった局所的治療介入のみでは、スギ花粉症の包括的な治療アプローチとしては不十分である可能性である。アレルゲン免疫療法を中心とした包括的かつ原因療法的な治療手段が重要であるものと考えられよう。
これらの症例が実際に気管支喘息を発症してゆくか、またかかる下気道炎症の制御あるいは喘息発症の阻止の上で、アレルゲン免疫療法が効果を発揮するか否かについては今後の重要な研究課題である。

F. 研究発表

1．論文発表
なし

2．学会発表
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G. 知的財産権の出願・登録状況(予定を含む)

1．特許取得
なし

2．実用新案登録
なし

3．その他
なし

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Changes in Airway Inflammation and Hyperresponsiveness after Inhaled Corticosteroid Cessation in Allergic Asthma

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Key Words

Airway hyperresponsiveness · Asthma, relapse · *Dermatophagoides farinae* · Eosinophils · House dust mites · Inhaled corticosteroids · Interleukin-4 · Remission, asthma

Abstract

Background: Most patients with asthma are currently controlled by pharmacotherapeutic means such as inhaled corticosteroid (ICS). However, whether ICS actually induces remission of asthma remains unknown. The present study evaluates changes in airway inflammation and hyperresponsiveness in adult patients with asthma after stopping ICS. ***Methods:*** We enrolled 11 patients with allergic asthma (7 males and 4 females; mean age, 52.3 years) who had been asymptomatic and had no exacerbation by low-dose ICS. Airway hyperresponsiveness (AHR) was assessed using methacholine challenge, and induced sputum was evaluated before and every 3 months after ICS cessation during the 1-year follow-up. ***Results:*** Among the 11 asthmatics, AHR increased in 10 (90.9%) and asthma clinically relapsed in 4 (36.4%) within 1 year of ICS cessation. AHR increased in all 7 asthmatics that were sensitized to *Dermatophagoides farinae* and asthma clinically relapsed in 4 (57.1%) of them. Furthermore, eosinophil numbers and IL-4 concentrations in the sputum significantly increased after ICS cessation. ***Conclusions:*** Remission with normal airway response to methacholine (no AHR) might be rare in adult patients with allergic asthma, and sensitization to house dust mites appears to play an important role in relapse. Therefore, ICS cessation should be carefully considered in patients sensitive to house dust mites. Serial determination of eosinophil counts or IL-4 concentrations in sputum might be appropriate for monitoring and preventing asthma relapse in adults.

Introduction

The prevalence of asthma is increasing, and effective pharmacological therapies such as inhaled corticosteroid (ICS) and other management strategies have been developed that have rendered asthma manageable. However, whether ICS could actually induce asthma remission (defined as the absence of asthma symptoms without asthma treatment [1]) was uncertain.

Asthma remission rates are considerably higher in childhood than in adults [2–12]. For example, Vonk et al. [2] reported that 22% of asthmatic children achieved complete remission [defined as the absence of asthma symptoms, no ICS use, normal lung function and no airway hyperresponsiveness (AHR)] and a further 30% achieved clinical remission (no symptoms and no ICS; total 52%) by the age of 32–42 years. In a study by Seker-

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el et al. [3], 27% and a further 26% of asthmatic children achieved complete remission (no symptoms, no medications, normal lung function and no AHR) and clinical remission (no symptoms and no medications), respectively, by the age of 17 years. Age at onset [4], disease duration [4], response to initial treatment [2, 5], forced expiratory volume in 1 s (FEV_1) at onset [2, 5–9], AHR at onset [6–9], total IgE [5–11], female gender [3, 6] or sensitization to house dust mites [8, 10] were predictive factors for remission or relapse of childhood asthma.

In contrast, little information is available regarding remission or relapse of adult asthma. Therefore, the prognosis of adult asthma is not understood in detail, and how treatment could be reduced or stopped has not been fully clarified. The Global Initiative for Asthma guidelines recommend that therapies for asthma control, e.g. ICS, can be stopped if asthma remains controlled in patients on the lowest dose and symptoms do not recur for 1 year [12]. However, the guidelines also indicate that little experimental data support the optimal timing, sequence and magnitude of reductions or cessation of treatment for asthmatic adults [12].

The present study investigates whether ICS administration could be stopped in adult asthmatics without relapse or AHR induction. We also evaluated changes in airway inflammation and AHR after ICS cessation.

Patients and Methods

Patients and Study Protocol

This observational study was conducted in the Allergy and Asthma Center of the Saitama Medical University Hospital after receiving approval from the Institutional Review Board of the Hospital and written informed consent from all patients who participated. Adult asthmatics that had been asymptomatic by low-dose ICS (fluticasone propionate 100 μg b.i.d. or equivalent) were evaluated for 12 months after ICS cessation. Asthma was diagnosed based on a history of recurrent wheezing, dyspnea, chest tightness and either reversible airflow limitation (FEV_1 <70% of predicted or a previous best value that increased by >15% after the inhalation of 200 μg salbutamol) or methacholine (MCh)-induced AHR. Before enrolment, we confirmed that the patients had been regularly treated with ICS for at least 6 months without regular long-acting bronchodilators, and that they had been asymptomatic and stable on low-dose ICS over the past 3 months. Eleven adult patients (7 males and 4 females, mean age 52.3 years, range: 30–71 years) participated in the study. All were atopic according to positive skin prick tests or had specific IgE antibody in response to common inhalant allergens such as house dust mites (*Dermatophagoides farinae, Df*). None of the patients had any other respiratory or systemic disease.

At the start of the run-in period, all of the patients recorded symptom scores and peak expiratory flow rates (PEFR) using a diary. At the end of the run-in period, all of them underwent baseline spirometry, the MCh challenge test and sputum evaluation for inflammation. To proceed to ICS cessation, asthma had to be 'controlled'. We defined 'controlled' asthma based on the definition of 'totally controlled' asthma in the Gaining Optimal Asthma ControL (GOAL) study [13] except for the evaluation of 'morning PEF'. (We used 'over 80% personal best' criteria, but not 'over 80% predicted' criteria [13].) After treatment cessation, all patients underwent the MCh challenge test and sputum evaluation every 3 months, and PEFR was monitored and symptoms were managed for 12 months. Relapse was defined as insufficient asthma control according to the GOAL study [13].

Monitoring PEFR and Asthmatic Symptoms

All of the patients monitored PEFR using a mini-Wright Peak Flow Meter twice daily (early morning and at bedtime before drug inhalation) for 3 consecutive months at the start of the study (run-in period) and then for up to 12 months. The weekly mean of the highest value of three exhalations into the meter (mean PEFR) was recorded in their diaries and evaluated. All patients also recorded asthmatic symptoms (cough, dyspnea and wheezing) in their diaries.

Pulmonary Function and MCh Challenge Tests

Pulmonary function tests were performed according to the American Thoracic Society guidelines [14] using an AS307 spirometer (Minato Medical Science, Osaka, Japan) and the MCh challenge proceeded as described previously [15, 16]. Briefly, baseline FEV_1 was recorded and then, after confirming that FEV_1 did not fall with an inhalation of saline, increasing concentrations of MCh (39–20,000 μg) were delivered using a DeVilbiss 646 jet nebulizer every 2 min, and FEV_1 was measured immediately after each inhalation. The concentration of MCh that induced a 20% fall in FEV_1 was calculated from the semi-log scale dose-response curve and is expressed as PD_{20} FEV_1 (cumulative dose producing a 20% fall in FEV_1).

Sputum Induction and Processing

Sputum was induced as described previously [17–19]. Salbutamol was delivered using a metered dose inhaler. Fifteen minutes later, sterile hypertonic saline (4.5%) was inhaled using an ultrasonic nebulizer at room temperature. Sputum was collected at 5-min intervals for up to 30 min. Rinsing with mouthwash before each expectoration was encouraged to minimize salivary contamination. All initial samples were discarded. Induced sputum samples collected into 50-ml polypropylene tubes were stored at 4°C for processing. Hanks' balanced salt solution (HBSS; 1 ml) containing 1% dithiothreitol (Sigma, St. Louis, Mo., USA) was added to sputum samples, which were then vortex mixed and repeatedly aspirated at ambient temperature until the mixture was homogeneous. Samples were diluted with HBSS to 5 ml, and separated by centrifugation at 400 *g* for 10 min. Cytokine concentrations were measured in supernatants, and cytospin slides of resuspended pellets were stained with May-Giemsa for differential cell counts. At least 500 inflammatory cells were counted for each sample. Cytospin slides were judged as adequate when <50% of squamous epithelial cells were present. Concentrations of IFN-γ and IL-4 in sputum supernatants were measured using Bio-Plex assay kits (Bio-Rad, Mississauga, Ont., Canada).

Table 1. Clinical characteristics of the patients

Patients			Disease duration years	FEV_1 % of predicted	Total IgE IU/ml	Specific IgE Ab to mite	Pets	Pediatric asthma	Smoking
No.	sex	age years							
1	F	67	7	105	33	–	dog	–	–
2	M	60	14	91	73	–	–	–	–
3	M	71	15	66	985	–	cat	–	–
4	F	64	17	89	188	+	cat	–	–
5	M	34	31	66	364	+	–	+	–
6	M	59	14	72	639	+	–	–	+
7	F	44	6	98	396	+	cat	–	–
8	M	30	1	112	524	+	–	–	–
9	M	63	23	72	67	–	cat	–	–
10	M	53	27	85	32	+	–	+	–
11	F	30	10	94	1,443	+	–	–	–

Table 2. Outcome after ICS cessation

Patient No.	PD_{20}, μg/ml		Recurrence
	before	after	
1	>20,000	312	–
2	>20,000	<78	–
3	154	78	–
4	3,375	ND	+ (10)
5	624	ND	+ (4)
6	1,812	ND	+ (16)
7	>20,000	4,750	–
8	>20,000	18,500	–
9	1,125	468	–
10	2,375	ND	+ (12)
11	2,812	1,875	–

ND = Not done. Mean duration of asthma recurrence after ICS is shown in parentheses (in weeks).

Statistical Analysis

Statistical significance was determined using the Kruskal-Wallis test. $p < 0.05$ was considered to indicate a statistically significant difference. Values are shown as means ± SEM.

Results

Treatment with ICS was discontinued in 11 patients with asthma that was 'controlled' by a minimal dose of ICS (fluticasone propionate 100 μg b.i.d. or equivalent) for >3 months. All of them had allergic asthma, 9 had adult-onset and 2 had a history of pediatric asthma. Ten were nonsmokers, 1 currently smoked, and 1 had used a short-acting β_2-agonist within the past year. In 8 patients, the duration of asthma was >10 years and FEV_1 was <70% of predicted in 2 of them (table 1).

Within 1 year of stopping ICS, AHR worsened in 10 (90.9%) of the 11 patients and asthma recurred in 4 (36.4%) of them (table 2). Asthma symptoms recurred in some patients immediately after ICS cessation and in all of them within 4 months. All of those with clinical asthma relapse were sensitized to *Df*, and the 1 current smoker as well as the patient who had used the short-acting β_2-agonist within the past year also relapsed. Of the 7 patients with specific IgE antibody against *Df*, AHR aggravated in all of them and asthma recurred in 4 (57.1%). AHR worsened in all 5 patients with a pet (dog or cat). Four patients without AHR during the run-in period have remained asymptomatic for 1 year (tables 1, 2).

Sputum was obtained from 9 patients. After ICS cessation, AHR worsened in 8 of them and 3 relapsed. The ratio of eosinophils in sputum obtained immediately before asthma relapse or AHR induction was significantly increased compared with that obtained during the run-in period (2.1 ± 0.6 vs. 1.1 ± 0.3%, $p < 0.05$; fig. 1). Macrophages, neutrophils, lymphocytes and total cell counts in sputum did not significantly differ (data not shown).

We measured cytokine concentrations in the sputum from 6 patients. After ICS cessation, AHR worsened in all patients and 2 relapsed. The IFN-γ concentration did not significantly change after ICS cessation (before vs. after cessation: 46.1 ± 10.6 vs. 34.6 ± 6.1 pg/ml; fig. 2). In contrast, the IL-4 concentration in sputum obtained just before asthma relapse or AHR induction was significantly increased compared with that during the run-in period (2.8 ± 0.8 vs. 1.5 ± 0.5 pg/ml, $p < 0.05$; fig. 2).

Among other potential predictors of asthma recurrence, the influence of disease duration, ICS treatment duration, lung function (e.g. FEV_1 and PEFR) or eosinophils in peripheral blood were also investigated, but none was associated with clinical relapse.

Discussion

We demonstrated that among 11 adult patients with allergic asthma 'controlled' by low-dose ICS alone, ICS cessation induced an increase in AHR in 10 (90.9%) of them and asthma relapse in 4 (36.4%). The discontinua-