Conclusion

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

Transparency

Declaration of funding
This study was funded by Kyowa Hakko Kirin Co., Ltd.

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

Some peer reviewers receive honoraria from CMRO for their review work. The peer reviewers of this paper have disclosed that they have no relevant financial relationships.

Acknowledgment
Data presented in this article corresponds with the Kyowa Hakko Kirin study identifier 4679-0421. The authors thank the following clinics for their participation: Hiroyuki Sano, Hidehiro Nakadate, Kazuo Ishikawa, Mizuho Goto, Hokkaido; Fumio Sakaguchi, Harumi Seki, Sadamu Minami, Hideaki Koyama, Keisuke Uesugi, Shunsuke Takamatsu, Tokyo; Takao Shizuka, Seiji Sawaki, Tomohide Hoshikawa, Hiromi Wada, Takuma Yoshikawa, Naoki Katsumi, Kanagawa; Hiroyuki Tada, Yasuko Murakawa, Chiba; Emiko Shiba, Saitama; Toshio Otsubo, Tomoko Torii, Toshiya Fujimori, Atsushi Usami, Masahiko Adachi, Shizuoka; Hirofumi Morita, Tadashi Shiraki, Aichi; Akiko Tamaki, Haruhiko Masutani, Hiroshi Kikumori, Kenji Baba, Kenichi Shimada, Osaka". The authors also thank the Kyowa Hakko Kirin 4679-0421 study team for their contribution to this study.

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—Review—

Allergen Immunotherapy for Allergic Rhinitis

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Abstract

Subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT) are the 2 forms of desensitization for allergic diseases which are used internationally. Despite the lack of sufficient evidence of the mechanism, SCIT has been used in Japan. When SCIT is performed appropriately, it is effective for allergic rhinitis, as supported by high-level evidence, including the results of meta-analysis. However, its use in Japan has not become widespread due to various problems, including the risk of anaphylaxis. Therefore, attention is being focused on SLIT because it is easier to perform but may be similarly effective and has been extensively discussed and evaluated internationally. Comparison of SCIT and SLIT can only be done with some allergen, which has also been discussed extenesively. Many patients and physicians would welcome the use of SLIT in Japan, and its first use against pollinosis due to Japanese cedar pollen is planned for 2014.

(J Nippon Med Sch 2010; 77: 285–289)

Key words: subcutaneous immunotherapy, sublingual immunotherapy, regulatory T cell, pollinosis

Introduction

Allergic rhinitis (AR) is a typical type I allergic disease in the classical classification of Gell and Coombs and is recognized as a chronic lifestyle disease by the Japanese Ministry of Health and Welfare. It reduces quality of life (QOL) even if it is unlikely to cause death. The recent increase in prevalence has been marked for atopic dermatitis and asthma as well as for AR. It is not possible to achieve advances in the treatment of other diseases if AR (a simple type I allergy) cannot be controlled.

Because AR is caused by specific allergens, immunotherapy is an important form of treatment. However, immunotherapy is not widely used in Japan because of the risk of anaphylaxis and the high cost. Subcutaneous immunotherapy (SCIT) was first performed by Noon in 1911[1]. Therefore, this treatment has been used for nearly a century, and much evidence has been accumulated. The latest evidence about immunotherapy is considered in this editorial.

SCIT

SCIT is a treatment for allergen-specific allergic diseases (**Fig. 1**) which involves the subcutaneous injection of gradually increasing doses of the allergen, with the aim of decreasing the patient's responsiveness. SCIT is mentioned in the "Practical Guideline for Management of Allergic Rhinitis in

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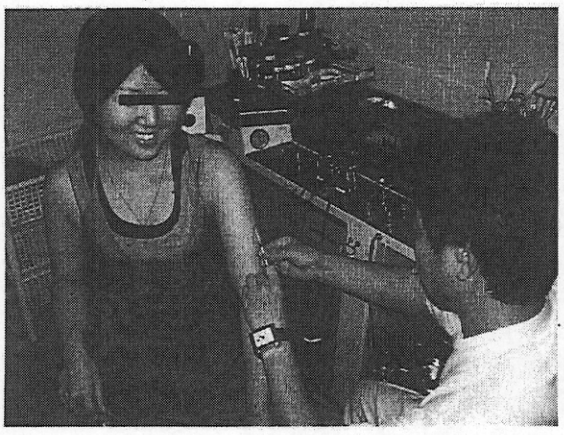

Fig. 1 SCIT.

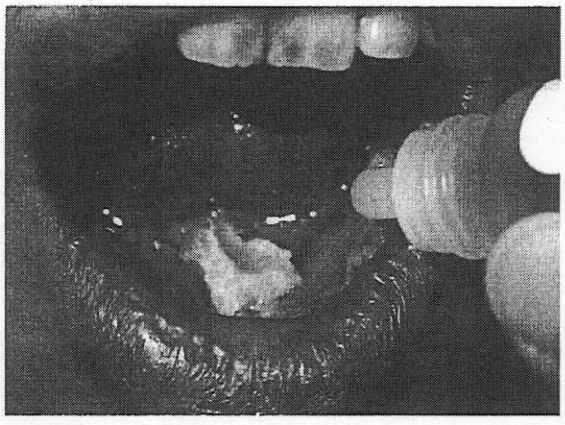

Fig. 2 SLIT.

Japan 2009"[2], and in "Allergic rhinitis and its impact on asthma (ARIA)"[3], which is an international guideline. The following features of SCIT were described in the immunotherapy opinion book published by the World Health Organization.

1. SCIT can be used for treatment of AR alone or with other methods.
2. SCIT is also effective for allergic conjunctivitis and allergic asthma.
3. SCIT should be performed by an allergy specialist.
4. Only standard allergens should be used.
5. The amount of allergen is increased gradually from a low dose until the maintenance dose is reached.
6. The maintenance dose is usually 5 to 20 μg of allergen per injection.
7. There is a risk of anaphylaxis, and appropriate precautions are necessary in case of emergency.
8. Treatment is generally continued for 3 to 5 years, although the optimum time is uncertain.

Efficacy, Mechanism, and Problems with SCIT

The subjective efficacy rate of SCIT for AR due to house dust (HD) mite is 80%, and the efficacy rate in the United States for the treatment of AR due to ragweed has been reported to be 90% or more[4]. In Japan, an efficacy rate of about 70% has been reported for pollinosis caused by Japanese cedar (JC) pollen, even allowing for seasonal variations in the pollen count. Persistence of the effect after the completion of treatment has been confirmed internationally[5].

An efficacy rate of 80% to 90% has been reported for atopic asthma with moderate symptoms[6,7]. Effectiveness for occupational allergy has also been reported.

SCIT is not considered effective for skin diseases, and it is not found in the guidelines for standard therapy despite some reports of its effectiveness in foreign countries.

There is considerable evidence for the efficacy of SCIT for bronchial asthma and AR in children[8]. Moreover, if SCIT controls pollinosis in infants, asthma can be prevented[9]. However, the frequency of an exaggerated immune response (anaphylaxis) to SCIT is greater in children than in adults.

Although the mechanism of action of SCIT has not been clarified, SCIT might depend on the suppression of IgE production[10,11], production of blocking antibodies[10-12], a decrease of mast cells in nasal mucosa[13], a decrease of cytokine production (down regulation of interleukin 5 mRNA)[14], and an increase of Th1 cytokines[15].

Thus, induction of tolerance or anergy, alteration of the Th1/Th2 balance, and regulation of T cells[16,17] may be involved in the immunologic mechanism of SCIT. However, the failure to completely clarify these mechanisms helps prevents the widespread use of SCIT.

The main problem with SCIT is the risk of systemic side effects. We have reported 17 cases of side effects in 1,642 patients with AR who underwent SCIT at the Department of Otolaryngology of Nippon Medical School from 1979

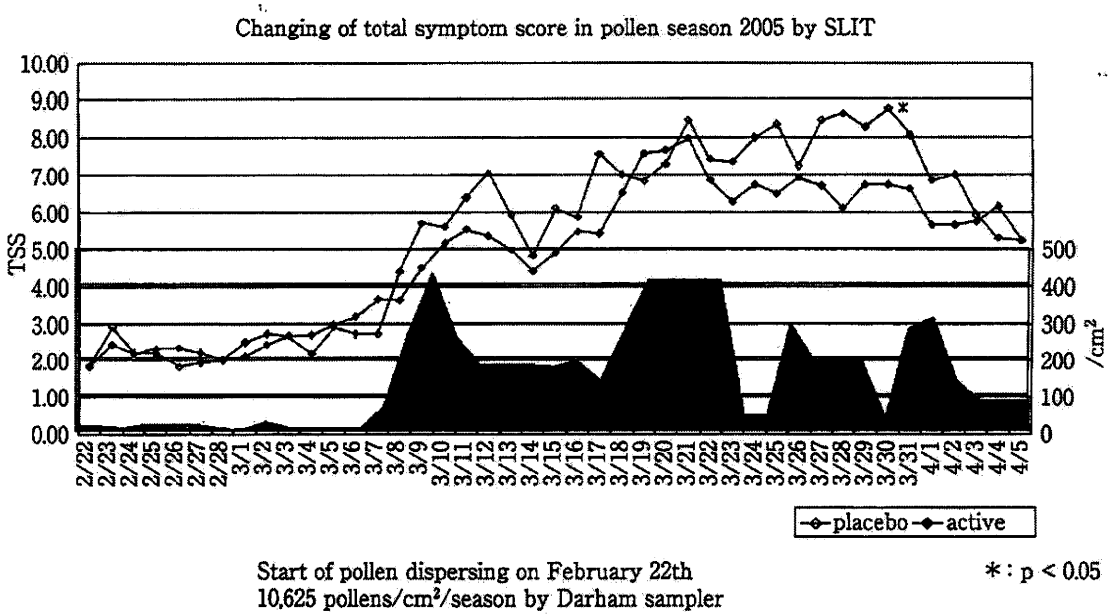

Fig. 3 JC pollen was present at high levels in 2005 (placebo-controlled study). The solid area shows the changes in the pollen count. Open squares represent the total symptom score (TSS) in the placebo group, and solid squares represent the TSS in the group given JC allergen.

through 1990. The clinical records in 5 of these cases were found to be incomplete. If these cases are excluded, the rates of eruptions, respiratory symptoms, and circulatory symptoms were 1% or less in the total patients base, which was 1/10th of the frequency in the total injection base[18].

SCIT and Sublingual Immunotherapy

Sublingual immunotherapy (SLIT) has been reported as an alternative form of immunotherapy. SLIT can affect numerous lymph nodes (oral lymph nodes, Waldeyer ring, and cervical nodes) and is more useful than SCIT because of its simplicity and safety (**Fig. 2**). A clinical trial of SLIT for JC pollinosis was started in our department because its efficacy has often been reported[19].

Two studies have compared the effects by means the double-dummy method, although few comparisons of SCIT and SLIT have assessed efficacy, cost, and other factors. Thirty-six patients with AR and asthma due to house dust mites were evaluated in the first randomized study in 1999[20]. Symptoms of asthma improved only with SCIT, but symptoms of rhinitis decreased with either SCIT or SLIT[20]. However, the asthma symptom score before immunotherapy was higher in the SCIT group than that in the SLIT group. In another study, 58 patients with silver birch pollinosis were randomly assigned to receive SCIT (n=19), SLIT (n=14), or a placebo (n=15). This study was small, but improvement in symptoms was significantly greater with SCIT or SLIT than with placebo but did not differ between the two immunotherapy methods; however, a difference might have been found if more cases were included[21]. Randomization was difficult because these studies had a limited number of subjects who differed in the severity of symptoms.

SLIT is already used widely for the treatment of AR, such as that due to pollinosis, in Europe. SLIT products for general clinical application are available from Stellergenes in France and Alk Abello in Denmark. There have been objections to SLIT in the United States, probably because SCIT used only by allergists However, a Cochrane cooperative meta-analysis by Wilson showed that SLIT was effective[22]. In the ARIA 2000 guidelines, SLIT is recommended for AR, conjunctivitis, and asthma due to pollen or mite allergy; these guidelines also clarified that SLIT influences the natural history of allergy in the same

way as SCIT[23]. The concept that the mechanism of SLIT involves IgX, which blocks all IgE, has been proposed[24]. However, a reported has pointed out the problem of presentation bias in a new meta-analysis. As a result, it has been suggested that there was no significant efficacy shown in some of the studies where SLIT was reported to be effective[25].

SLIT Research in Japan

Our group at Nippon Medical School began a clinical trial of SLIT for JC pollinosis in 2002[20]. Moreover, it is that the clinical trial of the doctor initiation was made as a high evidence in our group[26] and Chiba University[27], also. Our SLIT regimen involved sublingual JC allergen at a dose from 2 Japanese Allergology Units (JAU)/mL to 2,000 JAU/mL, with a daily increase over a 4-week period. The maximum dose (2,000 JAU/mL=20 drops) is administered twice during week 5. From week 6, the same dose is administered once a week through the season.

The level of JC and Japanese cypress pollen was about 12,000 grains/cm^2, which is high, during a 2005 study in Tokyo[26] Sixty patients were randomly assigned to each treatment, and a double-blind placebo-controlled examination was done. In patients with JC pollinosis, SLIT decreased the symptom score and the QOL score more than did placebo, and the improvement was by double rate compared to the placebo, especially in QOL (**Fig. 3**). The effect against JC pollinosis has also been studied by Chiba University and was found to be similar, with a decrease in JC-specific T cell clones in the peripheral blood[27]. Moreover, Tr1 (which induces regulatory T cells) was induced by SLIT in a study by Nippon Medical School and Mie University. The induction of regulatory T cells is a significant effect of SLIT[28].

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(Received, September 6, 2010)
(Accepted, October 21, 2010)

厚生労働科学研究費補助金（免疫アレルギー疾患等予防・治療研究事業）
分担研究報告書

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

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研究要旨

リアルタイムで花粉測定が可能な自動花粉測定器の活用は期待されてはいるものの、これまでの検討では自動花粉測定器には空中浮遊粒子との識別に問題があること、機種によっては感度に大きな問題あることが明らかになった。一方、大気中の全粒子の測定値と、その中で花粉と識別した粒子の両方の表示が可能な自動花粉測定器を用いて、自動花粉測定器の課題である空中非花粉粒子との識別能の向上について検討を進め、花粉見逃し率、花粉誤認率を算定して、補正を加えることで自動花粉測定器の精度向上が図れないかの検討を継続した。その結果、本年は大量のスギ花粉飛散がみられたが、自動花粉測定器による測定値に補正式を加えることで、相関係数の向上が確認された。また、抗原特異的なメモリーT 細胞クローンの測定方法について検討を進めたが、ダニに対する特異的メモリーT細胞についても測定を行い比較検討した。その結果、合成ペプチドを用いた特異的 T 細胞クローンの測定は、スギ花粉のみでなく、ダニについても可能であったがが、その動態には大きな違いが認められた。

A.　研究目的

リアルタイムで花粉測定が可能な自動花粉測定器の活用には期待が集まって、実際に様々な機種が使用されている。しかし、感度、特異性といった基本的な性能について十分な検討、評価がなされていないまま用いられているのが現状である。国内で従来から花粉測定に広く用いられてきた Durham 法は重力法であり、体積法の自動花粉測定器と直接測定数の比較は出来ないものの、相関については比較検討が可能である。これまで 6 年間にわたり Durham 法との詳細な比較検討を行ってきたが、自動花粉測定器には空中浮遊粒子との識別に問題があること、機種によっては感度に大きな問題あることが明らかになった。一方、大気中の全粒子の測定値と、その中で花粉と識別した粒子の両方の表示が可能な自動花粉測定器を用いて、自動花粉測定器の課題である空中非花粉粒子との識別能の向上について検討を進めてきた。すなわち、花粉見逃し率、花粉誤認率を算定して補正を加えることで自動花粉測定器の精度向上が図れないかの検討を行った。また、アレルギー性鼻炎の発症や治療効果の客観的な評価が可能なバイオマーカーの検出が求められているが、抗原特異的なメモリーT 細胞クローンの測定方法について検討を進めたが、本年度はダニに対する特異的メモリーT細胞についても測定を行い比較検討した。

B.　研究方法

測定全粒子数と花粉測定数の両値の表示が可能で、かつ比較的安価な自動花粉測定器（神栄）を用いて、2010 年 1 月の千葉市及び成田市での花粉非飛散期の測定データを利用して、花粉誤認率から補正マトリックスを作成した。この補正式を利用して本年のスギ花粉飛散期の自動花粉測定器による検出花粉数とダーラム式による花粉測定結果について相関を検討した。一方、スギ花粉の主要抗原である Cry j1 から 4 種類、Cry j2 から 3 種類のペプチドを選定してリジンでそれぞれのペプチドを連結させた合成ペプチドを利用して、またダニ主要抗原の Der f 1,2 からそれぞれ 7 種類のペプチドを作成、連結させて、スギ花粉症患者、ダニ通年性アレルギー性鼻炎患者末梢血中の抗原特異的 T 細胞のクローンサイズの測定を ELISPOT 法により行い、1 月と 5 月で比較した。

（倫理面への配慮）

アレルギー性鼻炎患者からの採血については、目的を十分に説明して同意を文書により得て実施した。また、試験計画は千葉大学の倫理員会に申請して許可を得た後に実施した。

C.　研究結果

自動花粉測定器神栄による本年 2 月 1 日から 3 月 31 日の間の成田市でのダーラム法による花粉測

定数（1 ㎠/day）は3137個であったが、神栄の測定値（1 ㎥/day）と日別相関係数は0.90、補正値は0.92であった。一方、千葉市での2月18日から3月30日のダーラム法による花粉測定数（1 ㎠/day）による積算値は2132で神栄との相関は0.77, 補正値では0.84であった。一方、合成ペプチドを用いたELISPOT法によるスギ花粉症患者末梢血リンパ球のスギ花粉特異的Th2メモリークローンサイズは1月に比較して5月には平均1.7倍の増加がみられ、スギ花粉特異的IgE値と相関がみられた。ダニアレルギー性鼻炎患者では全例にDer f特異的Th2細胞クローンが確認されたが、1月と5月でそのサイズに変化は見られなかった。

D. 考察：

本年は千葉市、成田市ともスギ・ヒノキ花粉飛散は例年以下であったが、自動花粉測定器による測定値に補正式を加えることで、相関係数の向上が確認された。合成ペプチドを用いた特異的T細胞クローンの測定は、スギ花粉のみでなく、ダニについても可能であるが、その動態には大きな違いが認められた。

E. 結論

自動花粉測定器に花粉見落とし率、花粉誤認率を考慮した新たな補正を加えることで、花粉測定の精度の向上が期待される。抗原ペプチドを用いた特異的T細胞クローンの測定は、比較的少量の採血からELISPOT法により簡便に測定が可能であり、今後、スギ花粉症の発症や寛解、免疫療法のバイオマーカーの検討に関する臨床応用が期待される。

F. 健康危険情報

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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

Original Paper

International Archives of Allergy and Immunology

Int Arch Allergy Immunol 2010;153:378–387
DOI: 10.1159/000316349

Received: June 20, 2009
Accepted after revision: March 8, 2010
Published online: June 18, 2010

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

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

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

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Introduction

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

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

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

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

Materials and Methods

Study Population

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

Clinical Protocols

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

Blood Samples

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

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

Antigens for in vitro Stimulation and ELISA

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

Antigen-Specific Immunoglobulin Titer

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

Flow Cytometric Analysis

For intracellular staining of Foxp3 and IL-10, PBMCs were cultured with or without Cry j 1 for 3 days, followed by culture with 10 ng/ml phorbol 12-myristate 13-acetate, 1 μM ionomycin and 2 μM monensin for 6 h. The PBMCs were stained with phycoerythrin-anti-CD25 (eBioscience, San Diego, Calif., USA) and phycoerythrin-Cy7-anti-CD4 antibody (BD Biosciences, San Diego, Calif., USA) in PBS containing 1% FCS and 0.1% sodium azide for 20 min at 4°C. After surface staining, the PBMCs were stained with FITC-anti-Foxp3 (clone PCH101, eBioscience) and allophycocyanin-anti-IL-10 antibody (BD Biosciences) for 30 min at 4°C using a Foxp3 staining buffer set (eBioscience) according to the manufacturer's instructions. The numbers of IL-10$^+$Foxp3$^+$ cells in 10^4 CD25$^+$CD4$^+$ leukocytes were calculated from the percentage of IL-10$^+$Foxp3$^+$ cells in CD25$^+$CD4$^+$ leukocytes.

Clinical Symptoms

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

Enzyme-Linked Immunospot Assay

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

Assay of Cytokine Production from PBMCs

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

Data Representation

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

Statistical Analysis

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

Results

Study Population and Adverse Events

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

Immunoglobulin Production

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

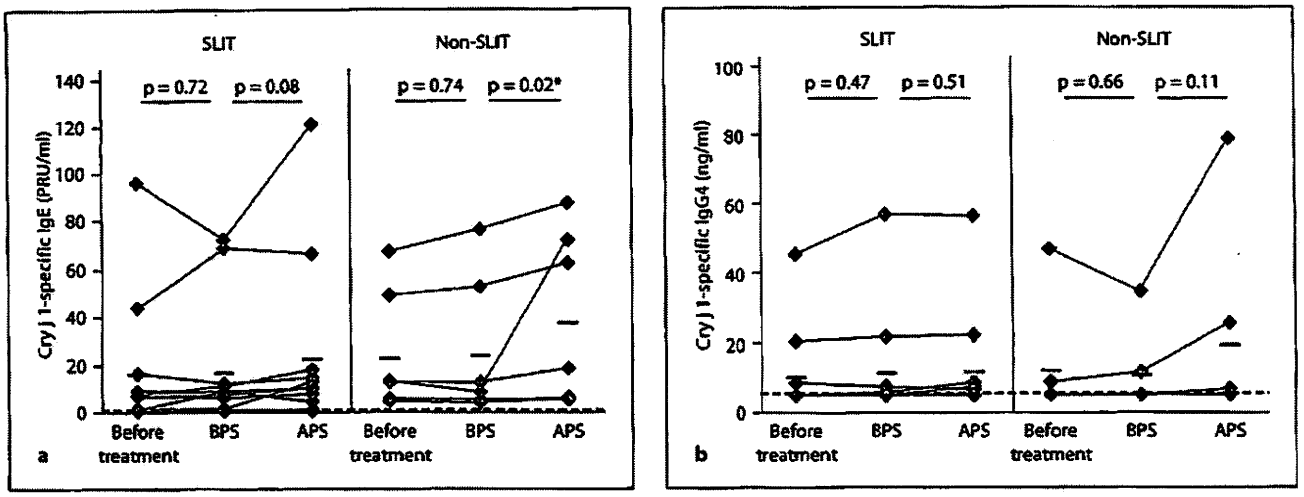

Fig. 1. Cry j 1-specific IgE (a) and IgG4 titer (b) from the SLIT and non-SLIT groups before treatment and before (BPS) and after the pollen season (APS). Bars show the group averages. The dashed line indicates the threshold for detection. PRU = Phadebas RAST unit. Statistical analysis was performed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

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

Division of the SLIT Group According to the Change in iTregs

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

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

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

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

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

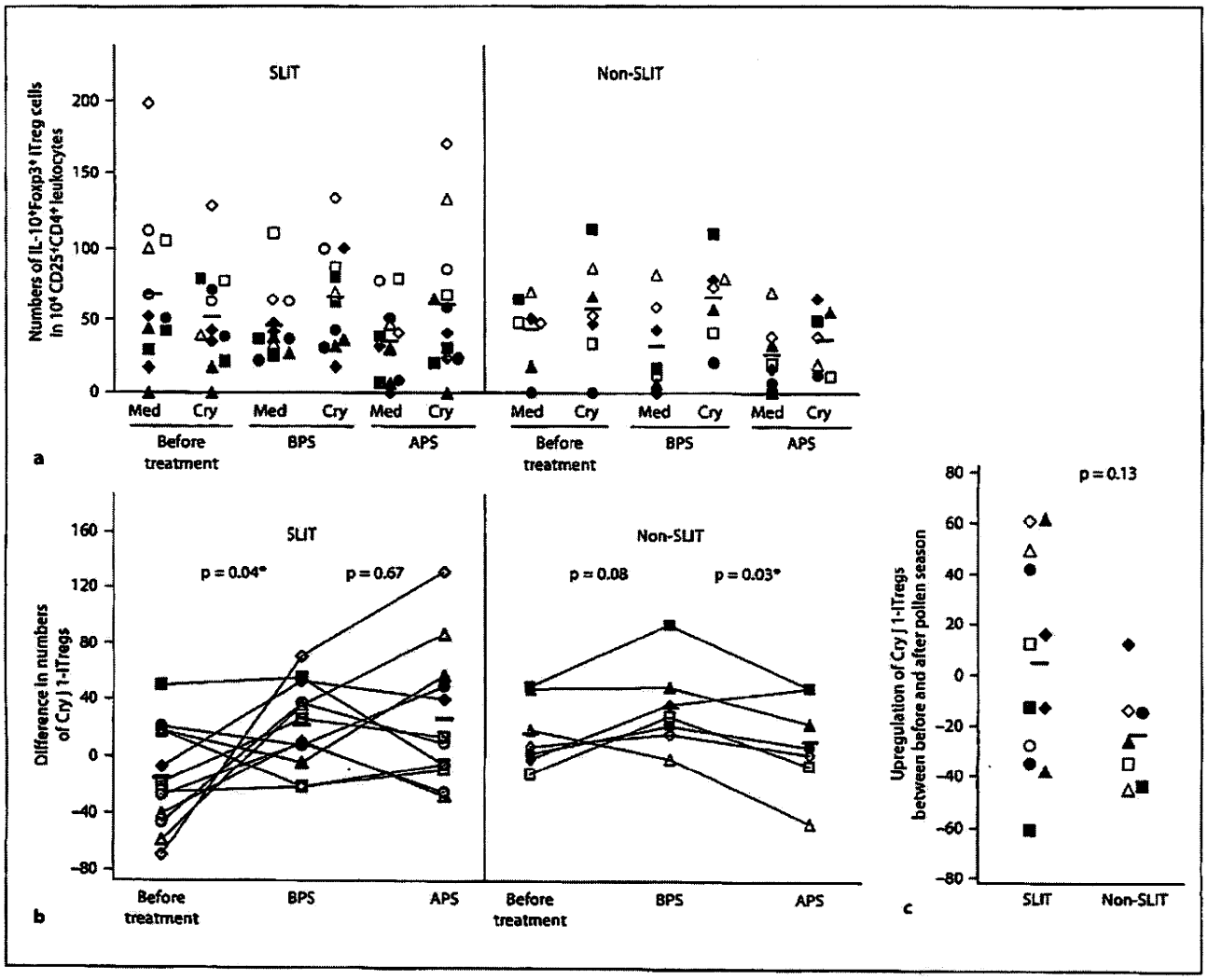

Fig. 2. **a** The numbers of IL-10$^+$Foxp3$^+$ cells in 10^4 CD25$^+$CD4$^+$ leukocytes (iTregs) cultured with (Cry) or without (Med) Cry j 1 before treatment and before (BPS) and after the pollen season (APS). Each symbol in the SLIT group and the non-SLIT group represents an identical individual.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

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

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

Th2-Type Cytokine Profiles

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

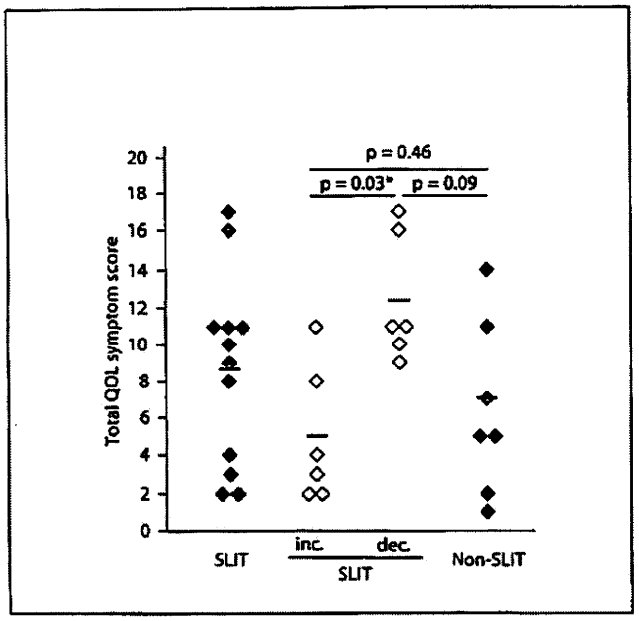

Fig. 3. Total symptom score from the QOL questionnaire was plotted for the SLIT and non-SLIT groups as well as for the subgroups from the SLIT group with increased (inc.) and decreased (dec.) iTregs. Bars show the group averages. Statistical analysis was performed using the Mann-Whitney U test. * $p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

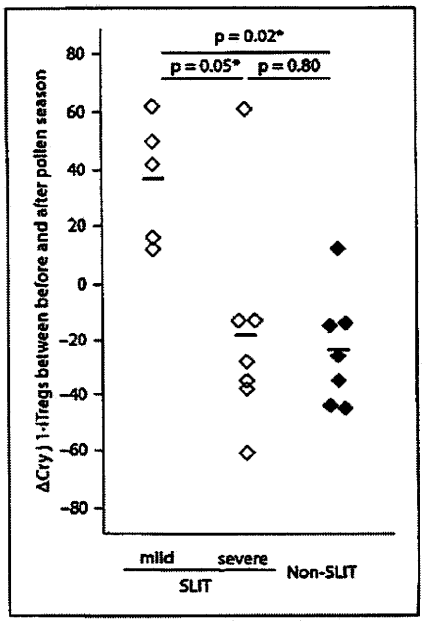

Fig. 4. The differences in numbers of Cry j 1-specific iTregs in 10^4 $CD25^+CD4^+$ leukocytes between before and after pollen season were plotted for the mild and severe subgroups of the SLIT group as well as for the non-SLIT group. The classification into severe and mild subgroups was based on the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

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

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

Discussion

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

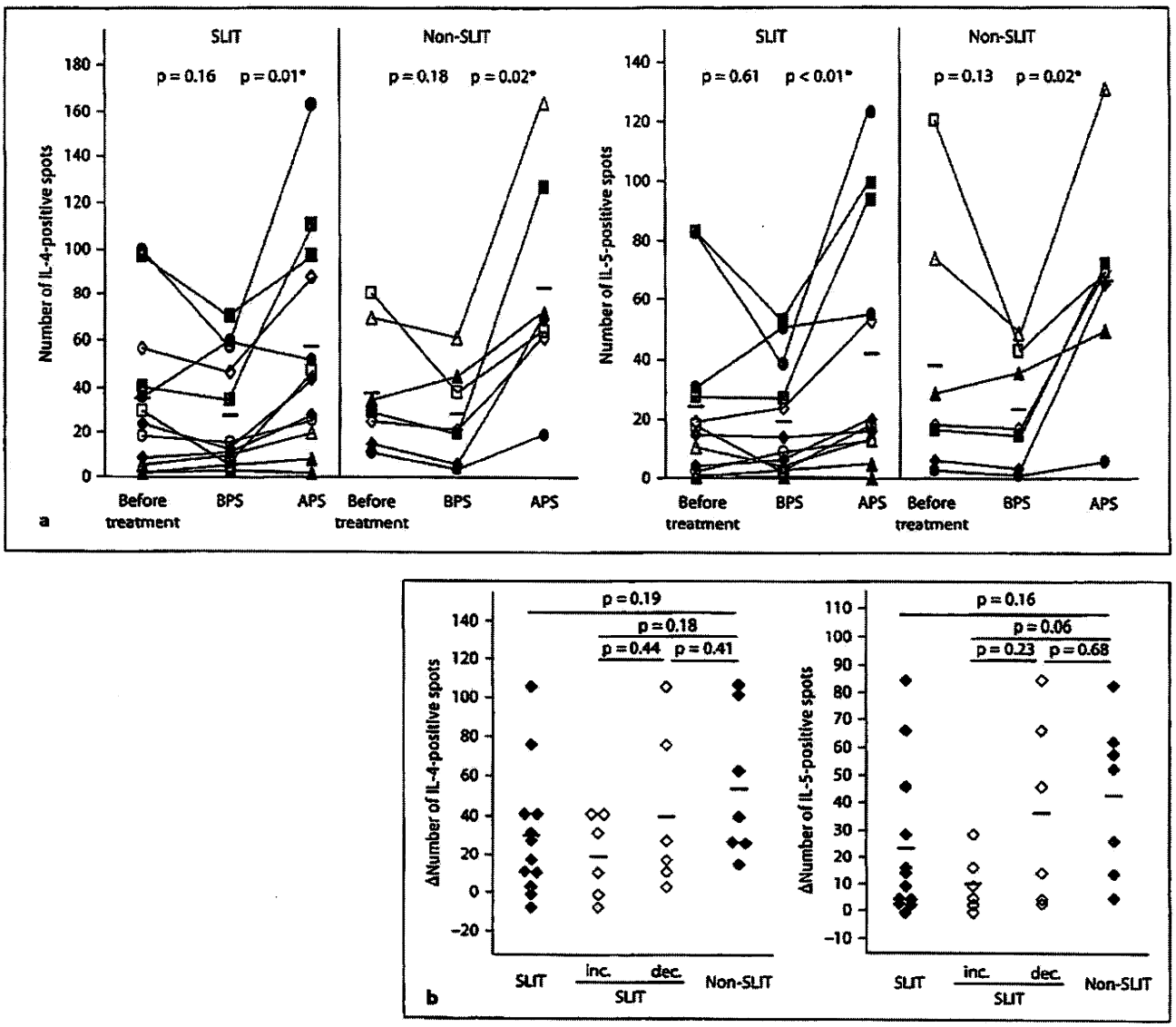

Fig. 5. **a** The numbers of Cry j 1-specific cytokine-producing cells before treatment and before (BPS) and after pollen season (APS). The numbers of positive cells for medium-only control were 1.3 ± 2.4 (SLIT) and 1.5 ± 3.1 (non-SLIT) for IL-4, and 1.0 ± 1.4 (SLIT) and 0.9 ± 1.6 (non-SLIT) for IL-5.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

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

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

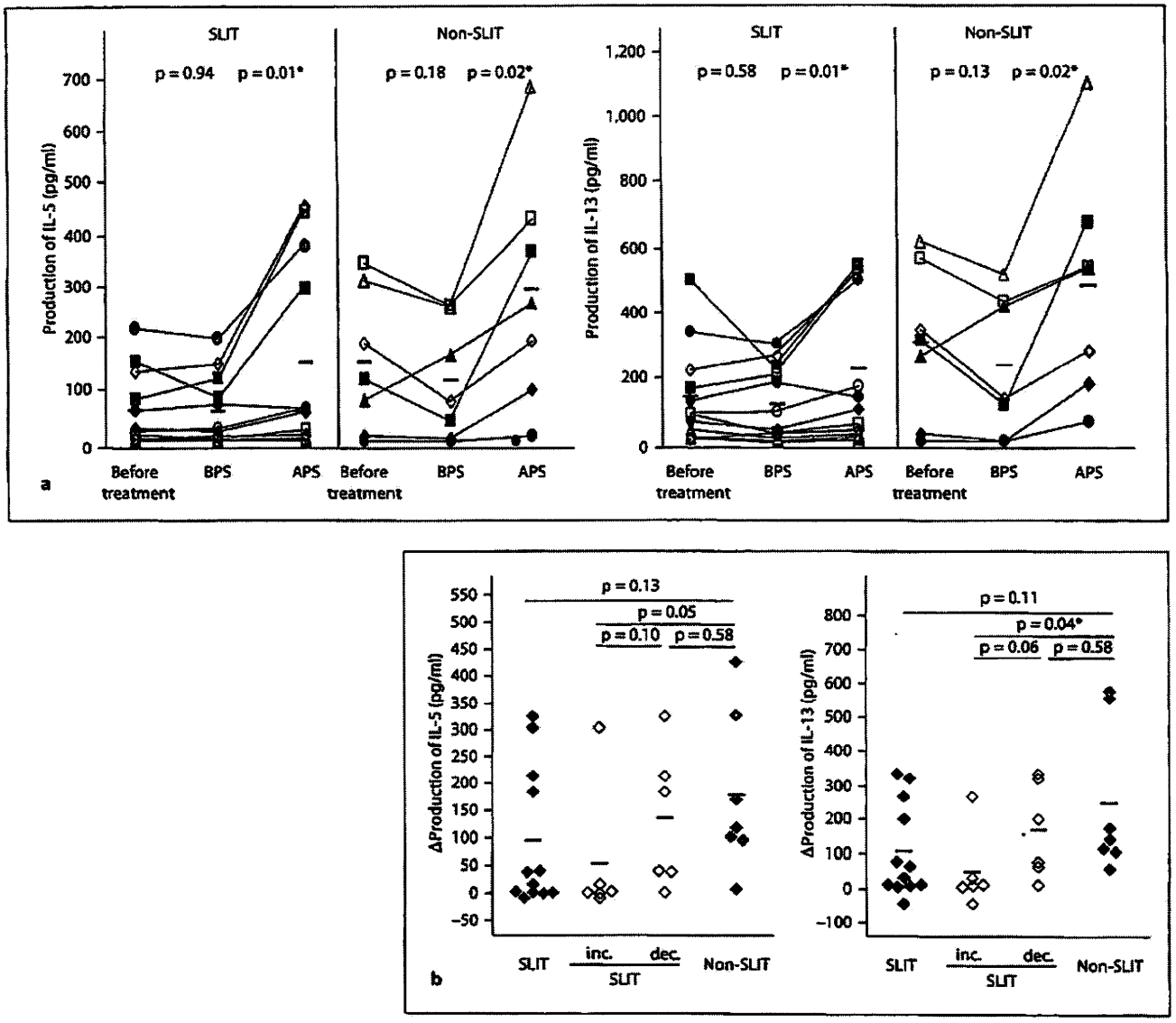

Fig. 6. **a** Th2-type Cry j 1-specific cytokine production before treatment and before (BPS) and after (APS) the pollen season. The cytokine production levels for medium-only control were 1.3 ± 2.0 (SLIT) and 3.4 ± 4.5 (non-SLIT) for IL-5, and 6.1 ± 9.6 (SLIT) and 10.6 ± 11.6 (non-SLIT) for IL-13. Bars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

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

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

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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Allergology International. 2010;59:21-31
DOI: 10.2332/allergolint.09-RAI-0151

REVIEW ARTICLE

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

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

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

INTRODUCTION

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

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

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Received 8 October 2009.

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

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

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

ANTIGENS FOR IMMUNOTHERAPY

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

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

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

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