Original article

Immunological parameters in prophylactic sublingual immunotherapy in asymptomatic subjects sensitized to Japanese cedar pollen

Kei-ichi Yamanaka, Said Ahmad Shah, Hiroshi Sakaida, Akisa Yamagawa, Sawako Masuda, Hitoshi Mizutani, Kazuhiko Takeuchi

*Department of Dermatology, Mie University Graduate School of Medicine, Mie, Japan
bDepartment of Otorhinolaryngology, Head & Neck Surgery, Mie University Graduate School of Medicine, Mie, Japan
cDepartment of Otorhinolaryngology, Mie National Hospital, Mie, Japan

A R T I C L E   I N F O

Article history:
Received 20 January 2014
Received in revised form 22 May 2014
Accepted 3 July 2014
Available online 20 October 2014

Keywords:
Immunotherapy
Interleukin-10
Mononuclear cells
Regulatory T cells
T cells

A B S T R A C T

Background: This study aims to examine the immunological parameters, focusing IL-10 productivity, in prophylactic sublingual immunotherapy (SLIT) in asymptomatic subjects sensitized to Japanese cedar pollen (JCP).

Methods: This study was conducted as part of a randomized, double-blind, placebo-controlled, multiple center trial, and was performed for two consecutive pollen seasons in 2012 and 2013. The present results were based only on our institution. We recruited 29 participants with specific IgE against JCP of at class 2 and higher levels without history of the pollinosis symptoms at the time of JCP scattering. The SLIT group received standardized JCP extract for five months over the pollen season. We observed and judged development of the symptoms in the pollen season. The percentage of IL-10 producing CD4+ T (Trl) cells, B cells and monocytes were analyzed by flow cytometry. JCP specific IgE and total IgE were also measured.

Results: The ratio of development of cedar pollinosis was significantly lower in the SLIT group compared to the placebo group in 2013. In 2012, the percentage of circulating Tr1 cells and IL-10 producing monocytes significantly increased in the SLIT group. In 2013, the percentage of circulating Tr1 cells and IL-10 producing B cells increased significantly in the SLIT group. The percentage of circulating IL-10 producing monocytes significantly decreased in the placebo group.

Conclusions: Prophylactic SLIT is effective for prevention of the development of pollinosis. Induction of IL-10 producing T cells, B cells and monocytes is an important mechanism of SLIT for prevention of pollinosis in asymptomatic but sensitized subjects.

Introduction

Japanese cedar pollinosis is an allergic disease specific to Japan with a high prevalence estimated to be 26.5%, which has increased by 10% over the past ten years. Seasonal allergic rhinitis induced by cedar pollen takes a chronic course in the majority of middle-aged patients. Remission rarely occurs, especially in the younger generation.

Sublingual immunotherapy (SLIT) is safer than conventional percutaneous antigen-specific immunotherapy, and is the only treatment which can completely cure the disease. It has been shown that SLIT is effective and safe in the treatment of cedar pollinosis by a randomized, placebo-controlled, double-blind study.

About 20% of asymptomatic subjects sensitized to this pollen develop symptoms in the pollen scattering season. Thus, it is important to prevent the development of pollinosis in these asymptomatic, sensitized subjects. To determine whether SLIT can prevent the development of pollinosis in sensitized subjects who have no history of pollinosis, a randomized, placebo-controlled, double blind trial was carried out over two pollinosis seasons in 2012 and 2013 in multiple facilities in Japan.

The mechanism of action of SLIT is not completely understood. However, IL-10 is critical for the induction of specific T cell tolerance and the increase in IL-10 production by monocytes and T cells during inflammatory responses or after SLIT may influence effector
cells involved in allergic responses. Increased IL-10 production following specific immunotherapy causes anergy in peripheral T cells, and regulates specific IgE and IgG4 production towards normal IgG4-related immunity. Low IL-10 productivity by monocytes and T cells is closely related to sensitivity to multiple allergens and resistance to allergic diseases. Augmentation of constitutive IL-10 production from the immune system is a potential therapeutic approach for allergic disorders. Thus we hypothesized that IL-10 may play an important role in prophylactic SLIT for asymptomatic sensitized subjects.

We also examined the ratio of specific IgE against total IgE (sIgE/total IgE), because the evidence concerning the relationship between this ratio and the efficacy of SLIT is conflicting. Di Lorenzo et al. reported that a high sIgE/total IgE ratio was associated with an effective response in sublingual and subcutaneous immunotherapy in monosensitized patients for the following allergens: grass, Parietaria judaica, Olea europea and house dust mite. On the other hand, Fujimura et al. reported that the sIgE/total IgE ratio before treatment correlated with the symptom-medication score in the SLIT group and that patients with low sIgE/total IgE ratios were more responsive to SLIT in treatment for Japanese pollinosis.

We examined the immunological parameters, including IL-10 productivity, in prophylactic SLIT in asymptomatic subjects sensitized to Japanese cedar pollen.

Methods

Study population

This study was conducted as part of a randomized, double-blind, placebo-controlled, multiple center trial in asymptomatic subjects sensitized to Japanese cedar pollen (JCP), and the present results were based only on our institution. The study was performed for two pollen seasons from December 2011 to April 2013. We recruited 29 participants with IgE specific to JCP of at least 2 class and higher without history of symptomatic pollinosis during JCP scattering. Japanese cedar pollen-specific IgE titers and total IgE in the serum were measured by CAP-FEIA (fluorescent enzyme immunoassay) (Phadia, Tokyo, Japan) before the study. Participants who were pregnant, breastfeeding or suffering from chronic rhinosinusitis were excluded.

Ethics statement

This study adhered to the tenets of the Declaration of Helsinki, and was approved by Mie University, Graduate School of Medicine Ethical Committee (No. 2283). A written informed consent was obtained from each subject before study.

Clinical protocols

The enrolled candidates were randomized into two groups by age and the levels of Cry j 1-specific IgE. The SLIT group received standardized JCP extract (Torii Pharmaceutical Co. Ltd., Tokyo, Japan), and the placebo group received an inactive placebo. The protocol consisted of treatments with graded courses of the extract in 50% glycerol, followed by maintenance therapy. Briefly, the extracts were graded in two concentrations: 200 and 2000 JAU/ml. From early December, the subjects received increasing doses beginning with 0.2 ml of the 200 JAU/ml vial and increasing by 0.2 ml every second day until reaching the maintenance dose of 1.0 ml of the 2000 JAU/ml for two weeks. From the third week, they received the maintenance dose of 1.0 ml of the 2000 JAU/ml daily until the end of April in the following year. The vaccine was taken sublingually, kept for 2 min without a retention reagent and then swallowed. The subjects in the placebo group received inactive 50% glycerol in saline.

Clinical symptoms and safety measurements

The subjects completed a pollinosis diary to record their nasal and eye symptoms and their use of symptom-reducing drugs. Development of the symptoms was determined on the basis of the pollinosis diary and a nasal provocation test performed at the end of April. The total amounts of pollen scattered from the Japanese cedar and Japanese cypress (Chamaecyparis obtusa) in Tsu city, Mie Prefecture, were 7031 and 16,578 grains/cm² during 2012 and 2013 pollen seasons, respectively.

Total and antigen-specific immunoglobulin titer

The levels of Cry j 1-specific IgE and total IgE in serum were measured by CAP-FEIA (fluorescent enzyme immunoassay) (Phadia, Tokyo, Japan).

Blood samples and PBMC culture

Peripheral blood was obtained from each subject before and after treatment (December and April) each year. Peripheral blood mononuclear cells (PBMC) were isolated from 10 ml of heparinized venous blood by density gradient centrifugation using Ficoll 1077 (Sigma, St. Louis, MO, USA). PBMC were cultured in RPMI 1640 medium (Nikken Bio Medical Laboratory, Kyoto, Japan) containing L-glutamine supplemented with 100 U/ml penicillin, 100 U/ml streptomycin (Invitrogen, Carlsbad, CA, USA) and 10% Human AB serum (Gemini Bio-Products, West Sacramento, CA, USA). Cells were plated onto 24-well tissue culture plates at a density of 2 × 10⁶ cells/ml/well and were incubated with 10 JAU of Cry j1 (Torii, Tokyo, Japan) for 8 h at 37 °C in an atmosphere of 5% CO₂. Endotoxin level was confirmed to be less than 0.1 ng/μg (1 EU/μg) of the protein in Cry j1.

IL-10 staining in T cells, B cells and monocytes

After 8 h cultivation with antigens, PBMC were collected and incubated with PE-conjugated IL-10 secretion assay kit according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA, USA). Cells were also co-stained with anti-CD4-FITC and CD19-PECY5 antibodies, or anti-CD14-FITC antibody (eBioscience, San Diego, CA, USA). The percentage of IL-10 producing CD4⁺ T cells, B cells and monocytes were determined using an Accuri C6 flow cytometer (Becton Dickinson, Mansfield, MA).

Statistical analysis

Two-group comparisons were performed using a Wilcoxon test or Mann–Whitney U-test to determine the significance of differences, or using an unpaired t-test as indicated. A p value of less than 0.05 was considered statistically significant.

Results

Clinical effects

Two subjects withdrew during the course of the study. The demographic characteristics of the 27 subjects before treatment are shown in Table 1. Clinical data from participants in 2012 and 2013 are shown in Table 2. As shown in Table 1, mean age of placebo group is higher compared to that of SLIT group; however, IL-10 production form T cells or monocytes is unchanged among...
younger generations (data not shown). In each year, 17 subjects participated in the study. Seven of the participants in 2012 continued to participate in the second year. These seven subjects did not develop symptoms in the first year.

In 2012, four of the SLIT group and one of the placebo group developed symptoms. There was no significant difference in the ratio of symptom development between the two groups in 2012. In 2013, seven of the placebo group but none of the SLIT group developed symptoms. The ratio of the development of pollinosis in the SLIT group was significantly lower than that of the placebo group in 2013 (p = 0.0098, Fisher's exact test). To clarify the possible factors influencing clinical effects, we compared age, sex ratio and slgE/tIgE ratio between 2012 and 2013. However, there was no significant difference in these factors in the two years (Table 2). We also compared these factors between the SLIT group and the placebo group in each year, but statistical significance was not detected (data not shown).

**Prognostic biomarker for clinical effects**

A comparison of the ratio of slgE/tIgE in the preseason period is shown in Fig. 1. The ratio of slgE/tIgE in the preseason was higher in those who developed symptoms in 2013 (B), but not in 2012 (A). In the SLIT group, the slgE/tIgE ratio in the preseason period was significantly higher in those who developed symptoms (C). In the placebo group, there was no significant difference in the ratio between the subjects developed pollinosis and those did not (D).

**IL-10 production in T cells, B cells and monocytes during SLIT**

In 2012, it was impossible for us to collect blood from one subject in each group due to their personal reasons. In 2013, one subject as the first year trial in the SLIT group and another subject as the second year trial in the placebo group were unable to come to the hospital for the blood collection in the postseason due to their personal reasons. Thus, we were unable to obtain blood samples from four subjects in total.

The percentage of IL-10-producing T cells, B cells and monocytes in 2012 is shown in Fig. 2. The percentage of circulating Tr1 cells (IL-10+ T cells) and IL-10-producing monocytes (IL-10+CD14+ cells) significantly increased in the SLIT group (p = 0.0117, Tr1 and p = 0.0117, monocyte, respectively). However, the number of IL-10-producing B cells (IL-10+CD19+ cells/CD19+ cells) remained unchanged. The results for the 2013 season are shown in Fig. 3. The percentages of circulating Tr1 and IL-10-producing B cells significantly increased in the SLIT group (p = 0.0277, Tr1 and p = 0.0277, B cell, respectively). On the other hand, the percentage of circulating IL-10-producing monocytes significantly decreased in the placebo group (p = 0.0077).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Clinical data of 27 participants at the start of the study.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>SLIT</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
</tr>
<tr>
<td>Number</td>
<td>13</td>
</tr>
<tr>
<td>Male/Female</td>
<td>5/8</td>
</tr>
<tr>
<td>Age (mean, range)</td>
<td>27.8 (18–47)</td>
</tr>
<tr>
<td>Total IgE (mean, range)</td>
<td>214 (23–823)</td>
</tr>
<tr>
<td>Specific IgE (mean, range)</td>
<td>13.9 (0.74–84.7)</td>
</tr>
<tr>
<td>Class (mean, range)</td>
<td>2.8 (2–5)</td>
</tr>
<tr>
<td>Ratio of slgE/tIgE (mean, range)</td>
<td>0.11 (0.0049–0.44)</td>
</tr>
</tbody>
</table>

1 Yates 2 × 2 Chi-squared test.
2 Student t-test.
3 Specific IgE to Japanese cedar pollen; ImmunoCAP raw value [UA/ml], mean.

The enrolled patients in 2013 were divided into two categories: the subjects with first year trial and the second year trial. Therefore the data of IL-10-producing T cells, B cells and monocytes in 2013 was calculated separately. As shown in Fig. 4A, nine subjects had participated as the first year trial. Three subjects in the SLIT group and six subjects in the placebo group are shown. IL-10-producing monocytes were statistically decreased in placebo group at the post season. The other hand, six subjects had participated as the second year trial. Three subjects in the SLIT group and three subjects in the placebo group are shown. Although the significant difference was undetected, the actual percentage of Tr1 and IL-10-producing B cells seem to be higher in the second-year trial compared to the first-year trial (Fig. 4B).

**Discussion**

To our knowledge, this is the first report examining the percentage of circulating IL-10 producing T cells, B cells and monocytes in prophylactic SLIT treatment in those who are sensitized to pollen but do not develop pollinosis. A significant increase in the percentage of Tr1 in the SLIT group was observed in both years of the study. The up-regulation of Tr1 cells may play a critical role in specific immunotherapy and be a useful marker of successful response in allergic rhinitis patients. It has been reported that the levels of allergen-specific Tr1 cells, IgG4 and allergen-induced IL-10 synthesis from PBMC cultures were significantly increased after cluster-specific immunotherapy in Der p-sensitized children with allergic rhinitis compared with baseline levels, with a significant correlation between increased levels of Tr1 cells and improvement in nasal symptoms.12

A significant increase in the circulating IL-10 producing B cells in the SLIT group was observed only in the 2013 season. IL-10-producing regulatory B cells suppress immune responses, and the lack of these cells leads to exacerbated symptoms. According to van de Veen et al., IL-10 producing B cells suppressed antigen-specific CD4+ T-cell proliferation.13 B cells specific for the major bee venom allergen phospholipase A2 (PLA) isolated from non-allergic beekeepers show increased expression of IL-10 and IgG4. IgG4 is a blocking antibody isotype with anti-inflammatory potential that is induced in human high-dose antigen tolerance.13 Furthermore, the frequency of IL-10+ PLA specific B cells increased in allergic patients receiving allergen-specific immunotherapy.14 IL-10–producing B cells, also known as regulatory B cells (Bregs), also play a key role in controlling autoimmunity. Mice lacking endogenous IL-10–producing regulatory B cells developed exacerbated disease and presented with a decrease in regulatory T cells.14

Monocyte is a major source of IL-10 in PBMC, and is a key cells for IL-10 mediated immunomodulation. The changes in percentage of IL-10 producing monocytes were different between the two
years. In 2012, the percentage of IL-10 producing monocytes significantly increased in the SLIT group. On the other hand, the percentage of IL-10 producing monocytes significantly decreased in the placebo group in 2013. Therefore a decrease in the percentage of IL-10 producing monocytes was prevented with the SLIT protocol. Pollinosis developed in 70% of the placebo group but none of the SLIT group in 2013. The percentage of IL-10 producing monocytes was significantly higher in healthy subjects than in those of IL-10 producing monocytes was prevented with the SLIT protocol. Pollinosis developed in 70% of the placebo group but none of the SLIT group in 2013. The percentage of IL-10 producing monocytes was significantly higher in healthy subjects than in those

Fig. 1. Comparison of the ratio of sIgE/tIgE in the preseason period. The ratio of sIgE/tIgE in the preseason was higher in those who developed symptoms in 2013 (B), but not in 2012 (A). In the SLIT group, the sIgE/tIgE ratio in the preseason period was significantly higher in those who developed pollinosis and those who did not (C). In the placebo group, there was no significant difference in the ratio between those who developed pollinosis and those who did not (D). When the subjects received SLIT for two years, only the data in the first year was adopted. The graph shows the mean ± standard deviation. The comparison was made using Mann–Whitney U-test.

Fig. 2. The percentage of IL-10-producing T cells, B cells and monocytes in 2012. The percentage of circulating Tr1 (IL-10⁺CD4⁺T cells/CD4⁺T cells) cells significantly increased in the SLIT group. The percentage of circulating IL-10 producing monocytes (IL-10⁺CD14⁺cells/CD14⁺cells) significantly increased in the SLIT group. However, the number of IL-10 producing B cells (IL-10⁺CD19⁺cells/CD19⁺cells) remained unchanged. (n = 8 in SLIT, n = 7 in placebo) The comparison between the pre-season values and the post-season values was performed using a Wilcoxon test. The comparison between the SLIT group and the placebo group was done using the Mann–Whitney U-test.
Fig. 3. The percentage of IL-10-producing T cells, B cells and monocytes in 2013. The percentage of circulating Tr1 (IL-10^+ CD4^+ T cells/CD4^+ T cells) cells and IL-10 producing B cells (IL-10^+ CD19^+ cells/CD19^+ cells) significantly increased in the SLIT group. The percentage of circulating IL-10 producing monocytes (IL-10^+ CD14^+ cells/CD14^+ cells) significantly decreased in the placebo group. (n = 6 in SLIT, n = 9 in placebo) The comparison between the pre-season values and the post-season values was performed using a Wilcoxon test. The comparison between the SLIT group and the placebo group was performed using the Mann–Whitney U-test.

Fig. 4. The percentage of IL-10-producing T cells, B cells and monocytes in 2013. (A) This figure shows 10 subjects who have participated in the first year. Three subjects in the SLIT group and six subjects in the placebo group are shown. IL-10 producing monocytes were statistically decreased in placebo group at the post season. (B) This figure shows 7 subjects who have participated in two consecutive years. Three subjects in the SLIT group and three subjects in the placebo group are shown. The significant difference was undetected. The comparison between pre-season values and the post-season values was performed using a Wilcoxon test. The comparison between the SLIT group and placebo group was performed using the Mann–Whitney’s U-test.
suffering Japanese cedar pollinosis. Thus from the result in 2013, it is assumed that decreased IL-10 production by monocytes is closely related to becoming symptomatic. On the other hand, SLIT prevented the subjects from becoming symptomatic by up-regulating the IL-10 productivity. Monocytes recognize antigens via Toll like receptors, digest antigen with intrinsic enzyme, and some populations such as CD14+ CD16− monocytes present antigens combined with expression of MHC class II like antigen presenting cells.12

The reason for the difference in the clinical efficacy of SLIT and the percentage of IL-10 producing cells between the two years is unclear. In 2012, nearly half of subjects received SLIT treatment developed the symptoms, although IL-10 producing T cells significantly increased in the SLIT group. Among patients with symptomatic pollinosis treated by SLIT, some patients do not show the efficacy, although Tr1 ratio is increased. In the same way, in 2012 we speculate increased level of Tr1 induced by SLIT was not enough to suppress nasal allergy symptoms. The difference in the exposed pollen amounts might have some effects to the results. Exposure to larger amounts of pollen in 2013 might have helped switching and expansion of IL-10 producing cells. It is known that high dose bee venom exposure in beekeepers by natural bee stings represents a model to understand mechanisms of T cell tolerance to allergens in healthy individuals.16 Rapid switch and expansion of IL-10 producing Tr1 cells and the use of multiple suppressive factors represent essential mechanisms in immune tolerance to a high dose of allergens in non-allergic individuals.16

The subjects who developed pollinosis in 2012 did not participate in the study the next year. Thus, the study in 2013 comprised new subjects (n = 10) and those who did not develop pollinosis in 2012 (n = 7). This might have influenced the clinical efficacy of SLIT and the percentage of the IL-10 producing cells.

Those who developed pollinosis in the SLIT group had a significantly higher sIgE/tIgE ratio which supports the results reported by Fujimura et al.13 We speculate that effector cells with a low specific IgE level are less likely to be activated by antigen crosslinking or are more susceptible to downregulation by IL-10 producing cells than those with a high specific IgE level. The symptoms of patients with a low sIgE/tIgE ratio may be more easily attenuated by suboptimal potentiation of IL-10 producing cells by SLIT.17 A high sIgE/tIgE ratio before the season may be predictive of development of polinnosis. Uekusa et al.14 examined 33 adults who were sensitized to JCP but who had not developed symptoms and found that the sIgE/tIgE was significantly higher before the season in the subjects who developed pollinosis.

A limitation of the present study is the small number of samples. Further study is necessary to perform the analysis using a larger number of subjects.

Prophylactic SLIT is effective in preventing development of pollinosis. IL-10 producing T cells, B cells and monocytes play important roles in the mechanism of SLIT for the prevention of pollinosis in asymptomatic and sensitized subjects.

Acknowledgments

We thank Ms. Rina Higashi for providing help and assistance with this study. This study was supported in part by a grant from the Ministry of Health, Labour and Welfare in Japan (Prevention and treatment of immunology and allergy disease; Chief: Yoshitaka Okamoto, H23-206) and by a discretionary budget allocation from the director of Mie University Hospital (2013). The Japanese cedar pollen extracts were provided by Torii Pharmaceutical, but this was approved by the Fair Trade Commission. The extracts were used for public clinical trial. There is no other financial relationship with any pharmaceutical companies including Torii Pharmaceutical regarding this study.

Conflict of interest

KY received a research funding from Torii Pharmaceutical, GSK, Daiichi-Sankyo, Kyowa Hakko Kirin. HM received a research funding from Torii Pharmaceutical. The rest of the authors have no conflict of interest.

References

6. Yamanaka K, Yuta A, Kakeda M, Kitagawa H, Oghara H, Gabazza EC, et al. SLIT improves cedar pollinosis by restoring IL-10 production from Tr1 and monocytes—IL-10 productivity is critical for becoming allergic—. Allergol Int 2011;60:45–51.