

Allergen-Induced Basophil CD203c Expression as a Biomarker for Rush Immunotherapy in Patients with Japanese Cedar Pollinosis

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Key Words

Rush immunotherapy · Basophil activation · CD203c · Histamine release · IgG₄ · Japanese cedar pollinosis

Abstract

Background: Rush immunotherapy (RIT) can confer rapid clinical benefit on patients with allergic rhinitis or asthma. However, biomarkers representing mechanisms for the efficacy of RIT are still to be established. CD203c is a basophil activation marker known to be upregulated by cross-linking of the FcεR1α receptor and may serve as a useful marker. **Objective:** We sought to investigate the changes in allergen-induced CD203c expression in patients with Japanese cedar pollen (JCP) pollinosis who received RIT. **Methods:** Nine patients treated with RIT were enrolled in the study. Whole blood was incubated with various concentrations of JCP extract. CD203c expression on basophils was quantitated by means of flow cytometry. JCP-specific IgG₄ levels in sera were measured with ELISA. Basophil histamine release, CAP-RAST to JCP (JCP-IgE) and total IgE were also examined. The biomarkers listed above were evaluated before and sequentially after RIT. Symptom and quality of life scores were obtained during pre- and posttreatment pollen seasons. **Results:** All patients showed significant improvement in symptom and quality of life scores after RIT. Serum JCP-specific IgG₄ titers were significantly elevated at 1 month and remained at high levels 12 months after the treatment. Stim-

ulation with JCP extract induced enhancement of basophil CD203c expression in a concentration-dependent manner except for 2 subjects in whom no increase in CD203c by an anti-IgE antibody was observed (nonresponders). Significant reductions in the responses were observed in 4 subjects after RIT (reduction in CD203c expression, RCE) whereas no changes were seen in 3 subjects (non-RCE). RCE subjects were older than non-RCE counterparts, with mean ages of 20 and 12 years, respectively. No significant changes in JCP-specific IgE and total IgE levels were seen before and after RIT. **Conclusion:** Allergen-induced CD203c expression in basophils may represent, at least in part, the cellular mechanism for the therapeutic responses to RIT for JCP pollinosis. However, further larger-scale studies to confirm the utility of the test are necessary.

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Introduction

During the past few decades, the prevalence of allergic diseases such as rhinitis and asthma has dramatically increased [1, 2]. Japanese cedar pollen (JCP) pollinosis represents a major clinical problem each year from late February to April in Japan, affecting as many as 10% of the Japanese population [3, 4]. Rush immunotherapy (RIT), in which a fast increment of allergen doses is allowed, has been reported to confer allergen-specific, rapid protec-

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tion from the disease. However, the mechanism for the early effects of RIT is still to be investigated. The short time frame of the treatment may be insufficient to induce alterations in T cell populations or changes related to T cell cytokine profiles. Changes in bioactivities of mast cells and basophils may be the first responses to the injected allergen [5].

Recently, flow cytometry-based tests for basophil activation status have been described to diagnose or confirm sensitization in allergic patients. CD63 can discriminate between resting and allergen-activated basophils [6–9]. Recent reports described an ectoenzyme, CD203c, as a more suitable basophil marker that is not only constitutively expressed on resting basophils but also upregulated at high levels on activated basophils [10–13]. CD203c belongs to a family of ectonucleotide pyrophosphatase/phosphodiesterases [14, 15] and has been described as being selectively expressed on basophils, mast cells and their CD34+ progenitors [12, 16]. As CD203c is rapidly upregulated after allergen challenge in sensitized patients, it has been proposed as a new tool for allergy diagnosis [10, 12]. In this report, we applied the CD203c measurement for monitoring the clinical course of RIT in patients with JCP pollinosis.

Materials and Methods

Subjects

Nine patients with JCP pollinosis participated in the study. The subjects included 6 females and 3 males, with a mean age of 16 years (range 6–38). They had elevated serum levels of specific IgE to JCP (CAP-RAST: median 112.2 IU/ml, range 58.4–294). None of the subjects had received specific immunotherapy previously. Written informed consent was obtained from the participants or the caregivers of those participants younger than 20 years. The study was approved by the ethics committee at Mie National Hospital.

Rush Immunotherapy

The patients were given subcutaneous injection of a standardized JCP allergen extract (Torii Co Ltd., Tokyo, Japan) with the following treatment regimen: day 1: 0.002, 0.006 and 0.02 Japanese allergy units (JAU); day 2: 0.06, 0.2, 0.6 and 2 JAU; day 3: 6, 20 and 40 JAU; day 4: 60, 80 and 100 JAU; day 5: 100 and 200 JAU; then a maintenance dose of 200 JAU was repeated on day 8 and 2 weeks from the start of treatment. Thereafter, the maintenance dose of the extract was given every 4 weeks. Blood sampling was performed before as well as 1 month, 2–3 months, 4–7 months and 10–14 months after the treatment. RIT was performed outside the pollen season. Antihistamines, short-acting β_2 agonists, oral corticosteroids and subcutaneous adrenaline were administered when systemic side effects occurred during RIT. Topical corticosteroids were given for local side effects.

Measurement with the Japanese Rhinoconjunctivitis Quality of Life Questionnaire (JRQLQ No. 1)

To evaluate the clinical efficacy of RIT on the nasal symptom severity (NSS) and disturbance of quality of life (QOLD), the disease-specific Japanese Rhinoconjunctivitis Quality of Life Questionnaire (JRQLQ) was used [17]. The degree of NSS/QOLD was scored as 1–4 in order (1 for a little and 4 for extremely severe or bothered). We evaluated the data from RIT patients before and after RIT during the pollen seasons.

Measurement of CD203c Expression on Basophils

A commercial kit (Allergenicity kit; Beckman Coulter, Fullerton, Calif., USA) was used for quantification of basophil CD203c expression. The test was performed according to the manufacturer's instructions. Briefly, EDTA-containing whole blood was incubated with various concentrations of the JCP extract (Torii) for 15 min after addition of a sufficient amount of calcium solution to override the chelating capacity of EDTA. Anti-IgE antibody at 4 $\mu\text{g/ml}$ as a positive control and PBS as a negative control were also used for stimulation. PC7-conjugated anti-CD3, FITC-conjugated anti-CRTH2 and PE-conjugated anti-CD203c antibodies were also added during the reaction. The samples were analyzed on a FC500 flow cytometer (Beckman Coulter). Basophils were detected on the basis of forward side scatter characteristics and expression of negative CD3 and positive CRTH2. Upregulation of CD203c on basophils was determined using a threshold that was defined by the fluorescence of unstimulated cells (negative control) and expressed as percentage of CD203c^{high}.

ELISA for JCP-Specific IgG₄

Each well of a 96-well microplate (Costar, Corning, N.Y., USA) was filled with 100 μl of JCP extract (Torii) at 100 $\mu\text{g/ml}$ in carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, 3.1 mM NaN₃, pH 9.6) and incubated for 18 h at 4°C. After removal of the antigen solution, the wells were treated with 150 μl of SuperBlock® blocking buffer (Pierce, Rockford, Ill., USA) for 1.5 h at 25°C. After washing 3 times with 200 μl /well of PBS-T (PBS containing 0.5 ml/l Tween 20), serum samples diluted with the blocking buffer were added to the wells, which were then incubated for 18 h at 4°C. Each well was washed 3 times with PBS-T, 100 μl of horseradish peroxidase-conjugated monoclonal mouse anti-human IgG₄ (clone HP6025; Zymed Laboratories Inc., South San Francisco, Calif., USA) diluted in the blocking buffer was added to each well and incubated for 1 h at 25°C. Following 5 times washing of the assay plates with PBS-T, the immunoreactivity was visualized by addition of 100 μl /well of substrate solution (TMB solution; KPL, Gaithersburg, Md., USA) for 15 min at 25°C. The reaction was stopped by the addition of 100 μl of 1 M hydrochloric acid to each well, and absorbance was measured at 450 nm using an immunoreader ARVO MX (PerkinElmer, Turku, Finland). The JCP-specific IgG₄ levels were expressed as arbitrary units against a positive serum pool, and experiments were performed in duplicate.

JCP-Specific IgE and Histamine Release Test

Serum levels of JCP-specific IgE were measured with CAP system (Phadra, Uppsala, Sweden). Basophil histamine release test was performed using a kit (HRT; Shionogi, Osaka, Japan) [18].

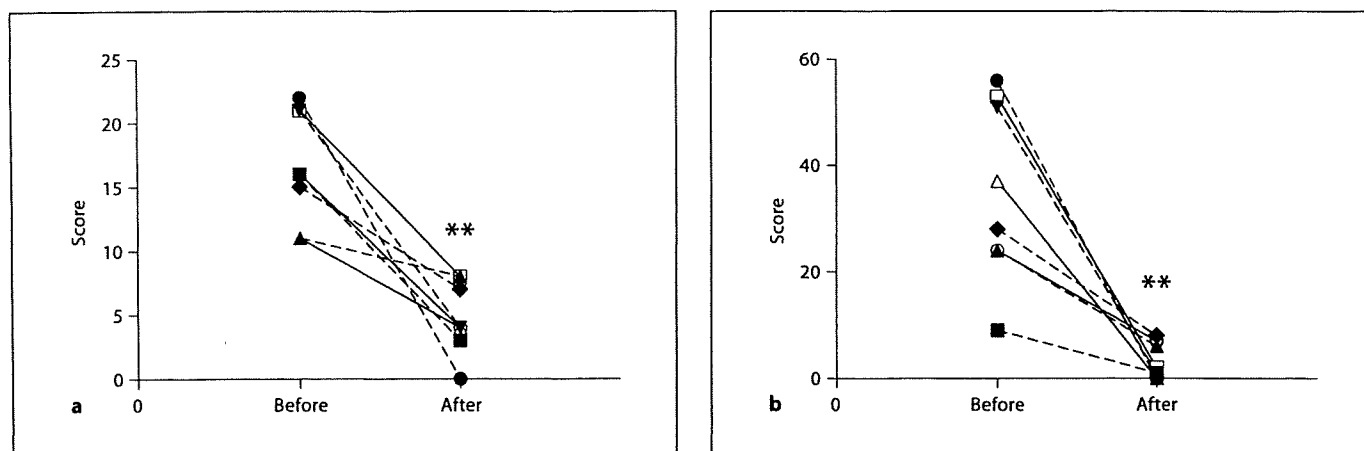


Fig. 1. Changes in symptom score (NSS; **a**) and quality of life score (QOLD; **b**) before and after RIT. Dotted lines represent patients who experienced the pollen season in 2005 before RIT and in 2006 after RIT. Solid lines represent patients who experienced the pollen season in 2006 before RIT and in 2007 after RIT. Total pollen counts in the Tsu area where the patients live are described in the Results section. ** $p < 0.01$, Wilcoxon's signed rank test.

Statistical Analysis

Wilcoxon's signed rank test for paired data was applied for NSS/QOLD score. The Kruskal-Wallis test and Dunn's multiple comparison test were applied for multiple comparisons and post hoc test.

Results

Improvement of NSS/QOLD Score after RIT

Both NSS and QOLD scores during JCP seasons were reduced after RIT in all patients (fig. 1), showing the clinical efficacy of RIT. Five patients experienced the pollen season in 2005 before RIT and in 2006 after RIT and 4 patients in 2006 before RIT and in 2007 after RIT. Total pollen counts in the Tsu area where the patients live were $11,502/\text{mm}^3$ in 2005, $2,643/\text{mm}^3$ in 2006 and $3,144/\text{mm}^3$ in 2007. It should be mentioned that the differences in pollen levels may have affected the symptoms, especially in the patients of the former group.

Changes in JCP-Specific IgE, IgG₄, HRT and CD203c Expression before and after RIT

There were no statistical changes in JCP-specific IgE levels after RIT (fig. 2a). In contrast, IgG₄ levels were significantly increased at 1 month after RIT, remained at elevated levels until 6 months, then slightly decreased at 1 year after the treatment (fig. 2b). There was no observable difference in HRT score before and after RIT (fig. 2c). The percentage of CD203c^{high} on stimulation with 0.3

$\mu\text{g}/\text{ml}$ of JCP extract tended to decrease at 1 month after RIT and continued to decrease until 1 year after the treatment, although the changes were not statistically significant (fig. 2d).

Subgroup of CD203c Responses

We then sought to characterize the CD203c responses by dividing the subjects according to the changes in JCP-induced CD203c expression after RIT. In 4 patients, dose-response curves for JCP-induced CD203c expression significantly shifted to the right and maximal levels of CD203c expression decreased after RIT [reduction in CD203c expression (RCE); a representative case is shown in fig. 3a]. On the other hand, patterns of CD203c expression showed no apparent changes after RIT in 3 patients [no RCE (NRCE); a representative case is shown in fig. 3b]. The remaining 2 patients were nonresponder phenotypes in whom CD203c expression did not increase with anti-IgE stimulation (fig. 3c.) [19, 20]. Table 1 summarizes the demographic data of the 3 groups. Although there were no statistically significant differences among the 3 subgroups in factors listed because of the small number of subjects, the RCE group had higher age and less prevalence of sensitivity to house dust mite. Age-dependent changes in allergen binding of IgE antibody have been reported [21] and the present results justify further investigation.

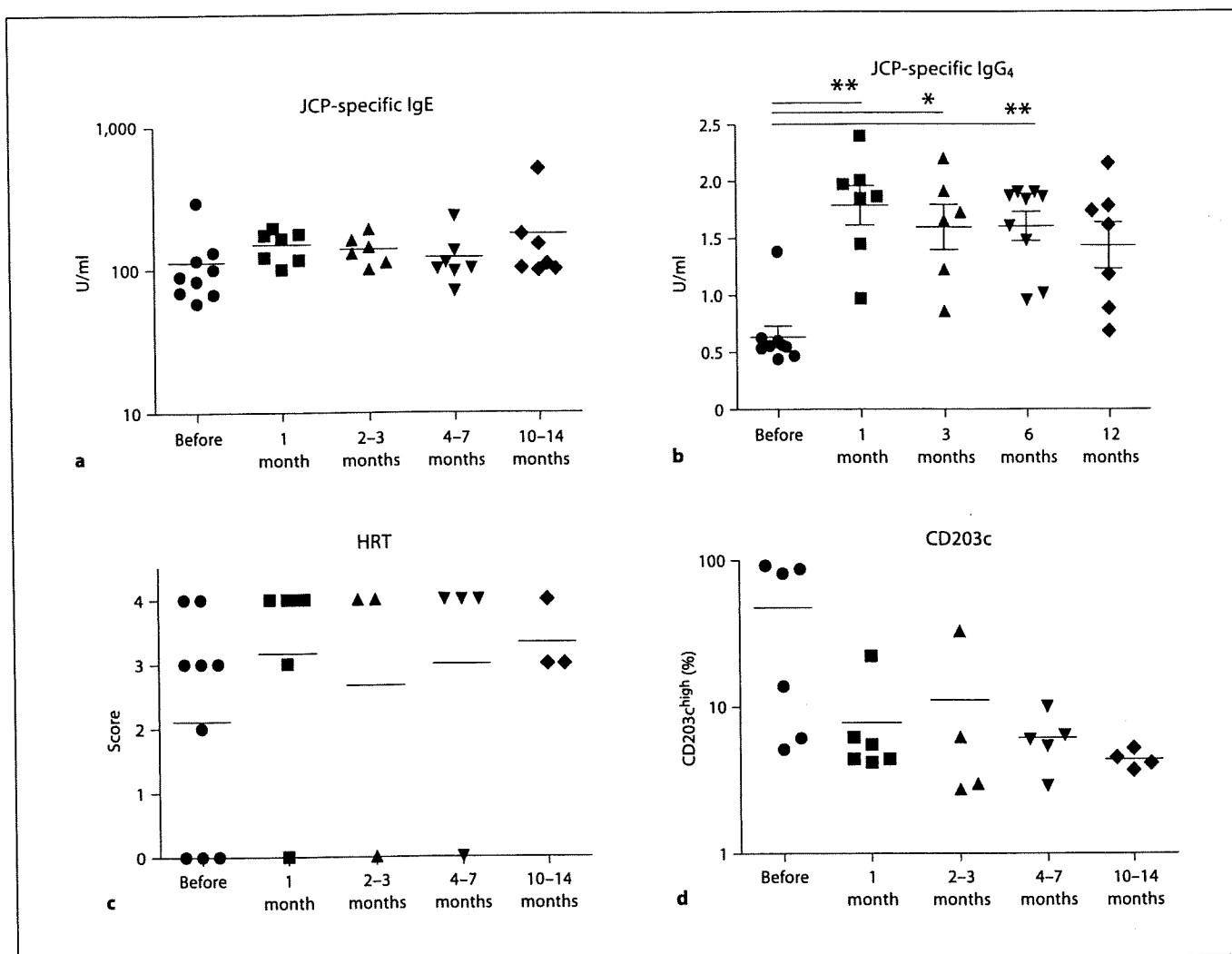


Fig. 2. Changes in JCP-specific IgE levels (a), JCP-specific IgG₄ levels (b), JCP-induced basophil histamine release score in HRT (c) and JCP-induced percentage of CD203c^{high} in basophils (d). * $p < 0.05$, ** $p < 0.01$, Dunn's multiple comparison test.

Table 1. Phenotypes of CD203c expression pattern before and after RIT

	RCE (n = 4)		NRCE (n = 3)		Nonresponders (n = 2)	
	before	after	before	after	before	after
Mean age, years	20 (13–38)		9 (6–11)		12 (11–12)	
Positive house dust mite-specific IgE, n	1 (25%)		2 (66.7%)		2 (100%)	
Total IgE, IU/ml	234 (173–1,083)	417 (364–1,415)	697 (154–7,260)	777 (568–7,732)	571 (521–620)	498 (483–513)
JCP-specific IgE, IU/ml	108	101	68	112	108	143
IgG ₄ , arbitrary units	0.587	1.546	0.556	1.742	0.520	1.365

Figures in parentheses are ranges, unless indicated otherwise.

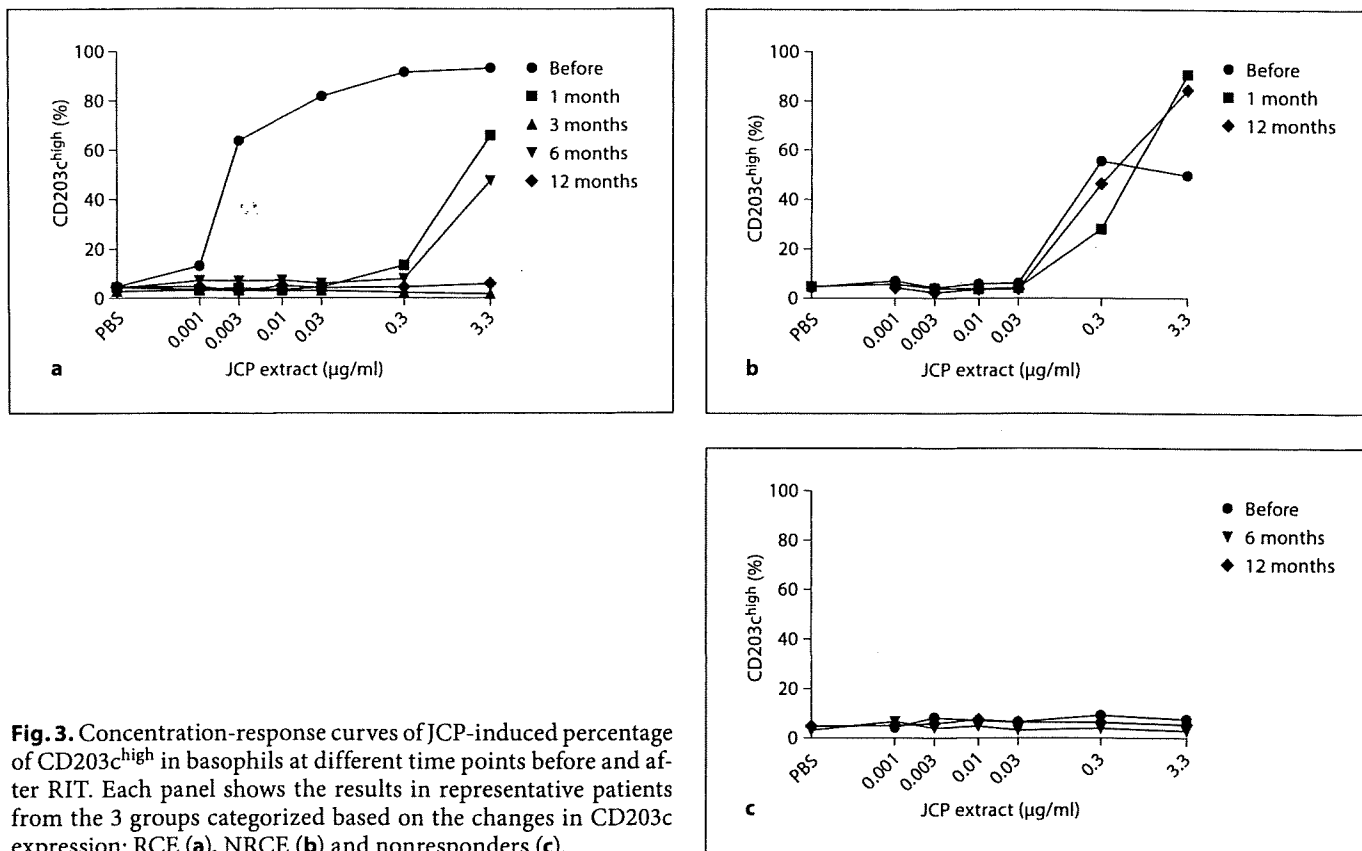


Fig. 3. Concentration-response curves of JCP-induced percentage of CD203c^{high} in basophils at different time points before and after RIT. Each panel shows the results in representative patients from the 3 groups categorized based on the changes in CD203c expression: RCE (a), NRCE (b) and nonresponders (c).

Discussion

In this study, we observed that RIT with JCP conferred significant improvement in symptom and quality of life scores in patients with JCP pollinosis. Along with the clinical improvement, serum levels of JCP-specific IgG₄ were significantly elevated as early as 1 month after treatment, whereas JCP-specific IgE and HRT showed variable changes. JCP extract-induced enhancement of CD203c expression tended to decrease after RIT. There were distinct phenotypes in terms of CD203c expression responses: the RCE group with significant reduction in JCP-induced CD203c expression after RIT and the NRCE group with no changes in the reaction.

Increase in specific IgG antibodies to allergens during immunotherapy has been well described. Usually, IgG₁ rises first, followed by IgG₄ that remains at high levels, whereas IgG₁ starts to decline after some time [22–24]. Our data, demonstrating significant increase in IgG₄ levels after RIT in JCP pollinosis, confirmed the previous observations in other allergen-specific immunothera-

pies. In separate experiments, we observed that the addition of the post-RIT serum significantly suppressed JCP-induced histamine release from passively sensitized basophils [25]. Importantly, we demonstrated that the serum before and after RIT had essentially similar sensitizing capacity and that the inhibitory activity of the post-RIT serum was heat resistant [25]. These results suggest that the suppressing effect on basophil degranulation may be attributed to so-called blocking IgG antibody generated in the patients, not to the changes in IgE antibody [25].

We demonstrated that allergen-induced expression of CD203c on basophils was also generally suppressed after RIT, although the changes were not statistically significant. On the contrary, HRT, a similar basophil activation test, showed variable results in this study. The CD203c assay employs whole blood during incubation with JCP allergen, which allows not only surface-bound IgE on basophils but serum factors or other circulating cells to affect activation status of basophils. On the other hand, HRT employs immunomagnetically purified basophils

during allergen stimulation [18] and represents only basophil-related factors such as density and/or avidity of membrane-bound allergen-specific IgE and intrinsic histamine-releasing activity of the cells. We suspected that disagreement in the 2 basophil activation tests may be due to the difference in the assay systems described above. Since in vitro blocking activity [25] and elevated JCP-specific IgG₄ in the post-RIT sera were clearly observed, it is reasonable to assume that detected immunological changes induced by the present immunotherapy took place, at least in part, in serum, possibly IgG₄ blocking antibody, not in basophils themselves or the surface-bound IgE.

The potency of blocking activity in vitro, however, varied among the patients [25], even though elevation of IgG₄ levels was found in all patients. Likewise, some of the patients in the present study did not exhibit post-RIT suppression in JCP-induced CD203c expression (RCE vs. NRCE shown in fig. 3a and b). In accordance with the findings, sera from RCE showed significant blocking activity in vitro, while those from NRCE patients did not (data not shown). Allergen-specific IgG directed against the same epitopes as IgE may result in direct competition for allergen binding and a blocking effect. It is possible that induction of IgG specific for other epitopes may result in a failure of the IgG capacity to compete with IgE, which can explain the variable results in this study.

Nonetheless, allergen-specific immunotherapy has been reported to induce T regulatory cells (Treg) [26]

producing TGF- β and IL-10 and the latter cytokine was shown to induce specific IgG₄ production [27]. In this context, the elevation of JCP-specific IgG₄ titers after RIT may be interpreted as a consequence of Treg induction. The lack of blocking activity in some patients, mainly seen in NRCE, could be substituted by other suppressive functions of Treg [28], leading to the significant clinical effect.

In conclusion, we measured allergen-induced CD203c expression in basophils as a marker for the therapeutic responses of RIT for JCP pollinosis. Although the results did not confirm that the basophil activation test is useful for monitoring all patients, the test surely represents certain aspects in the mechanisms of RIT. Further larger-scale studies are necessary to confirm the utility of the test for clinical evaluation.

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Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of the article.

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Blocking Antibody Is Generated in Allergic Rhinitis Patients during Specific Immunotherapy Using Standardized Japanese Cedar Pollen Extract

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Key Words

Basophils · Blocking antibody · Histamine release · Japanese cedar pollen · Specific immunotherapy

Abstract

Background: Japanese cedar pollen is by far the most important cause of allergic rhinitis in Japan. In this study, we assessed the induction of blocking antibody during specific immunotherapy (SIT) using a recently standardized allergen extract from Japanese cedar pollen. **Methods:** Basophils from nonallergic subjects were passively sensitized with serum samples prepared from pollinosis patients before and after SIT; all patients showed good clinical efficacy. The cells were then stimulated with the standardized allergen, and histamine release was measured. In most experiments, the basophil stimulation buffer contained 1% serum. **Results:** Pollinosis patients' sera obtained both before and after SIT showed essentially similar sensitizing capacity for basophils. Basophil degranulation in response to a relatively low concentration of pollen extract was effectively suppressed by addition of post-SIT serum samples, indicating the presence of blocking antibody. The blocking antibody was IgG, and its potency varied widely among the donor patients. **Conclu-**

sions: The standardized allergen extract from Japanese cedar pollen is useful not only for clinical application in SIT, but also for testing for induction of blocking antibody during SIT.

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Introduction

Recently, the prevalence of allergic pollinosis has increased strikingly in Japan. The most important allergen is Japanese cedar pollen: it induces severe rhinitis and conjunctivitis symptoms in the spring season in >20% of the population [1]. Allergic symptoms obviously hamper the patients' quality of life. Among the various current therapeutic strategies for this disease, allergen-specific immunotherapy (SIT) is the only antigen-selective, clinically applicable approach. Many studies have proven that SIT can generally alleviate clinical symptoms and antigen-induced responses in patients with bronchial asthma and rhinitis. In addition, the clinical efficacy of SIT can last for a long time even after repeated allergen inoculation is stopped. The effectiveness of SIT is known to involve multiple mechanisms, including induction of block-

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ing antibody, modulation of the Th1/Th2 imbalance and so on [2]. In order to expand our knowledge of those basic mechanisms underlying SIT, allergen identification and standardization are essential steps. In this study, we focused on the induction of blocking antibody by using a recently standardized allergen, a Japanese cedar pollen extract [3, 4]. We analyzed sera from Japanese cedar pollinosis patients who had undergone SIT, and found that those post-SIT sera contained blocking antibody which inhibited *in vitro* basophil histamine release in response to Japanese cedar pollen extract.

Materials and Methods

Reagents

The following reagents were purchased as indicated: dextran T500 and Percoll (GE Healthcare, Uppsala, Sweden); PBS (Gibco, Grand Island, N.Y., USA); PIPES (Sigma, St. Louis, Mo., USA); a standardized allergen solution containing Japanese cedar pollen extract [stock concentration: 2,000 Japanese allergy units (JAU)/ml; Torii, Tokyo, Japan]; calcium ionophore A23187 (Calbiochem-Behring, La Jolla, Calif., USA).

Buffer

PIPES-A buffer contained 25 mM PIPES, 119 mM NaCl, 5 mM KCl and 0.03% human serum albumin, and was adjusted to pH 7.4. For stimulation of basophils, PIPES-A containing 2 mM Ca^{2+} and 0.5 mM Mg^{2+} (PIPES-ACM) was used.

Serum Samples

Blood samples were taken from 5 consenting patients with Japanese cedar pollinosis before and after SIT using Japanese cedar pollen extract. All patients showed a good response to SIT. Serum samples were prepared by centrifuging the blood samples, and stored at -80°C until used. The patients' demographics and serum total IgE, pollen-specific IgE and IgG₄ levels were described in another paper by Nagao et al. [5].

Basophil Preparation

Human basophils were isolated from venous blood obtained from consenting volunteers with no history of atopic diseases. The basophils were semipurified by density centrifugation using Percoll solutions of different densities (1.080 and 1.070 g/ml), as previously described [6]. The mean purity of these Percoll-separated basophil preparations was 12.3%. In some experiments, venous blood was drawn from consenting subjects with cedar pollinosis who demonstrated positive results for serum IgE specific to Japanese cedar pollen. Leukocytes from these subjects were separated by dextran sedimentation as previously described [7]. The purity of these dextran-separated basophil preparations was approximately 1%.

IgE Stripping and Passive Sensitization of Basophils

Percoll-separated basophils from nonatopic subjects were treated at 4°C for 5 min with acetate buffer (pH 3.7) to remove surface-bound IgE [8]. After washing, the cells were resuspended

in PIPES-A buffer containing 4 mM EDTA and then mixed with an equal volume of a patient's serum. Passive sensitization was performed by incubation of the basophil preparations at 37°C for 2 h.

Histamine Release from Basophils

Histamine release was performed as previously described [7]. Briefly, cells were resuspended in PIPES-ACM buffer and then stimulated with the standardized allergen extract at 37°C for 45 min in polystyrene tubes. The supernatants were stored at 4°C until histamine assay by an automated fluorometric technique. Histamine release was expressed as a percentage of the total cellular histamine after subtracting the spontaneous release (consistently less than 7%) in the absence of secretagogues. Each experiment was performed at least in duplicate, and mean values were calculated.

In experiments analyzing for a blocking effect of serum on basophil histamine release, a 2-fold dose of cedar pollen extract was preincubated with a 2-fold concentration of serum sample in 200 μl of PIPES-ACM buffer for 30 min at 37°C . Then 200 μl of basophil preparation was added to each tube, and the cells were stimulated for 45 min.

IgG Purification from Sera

In some experiments, IgG was purified from pollinosis patients' post-SIT sera using an MAb Trap Kit (GE Healthcare), according to the manufacturer's instructions. This kit includes a protein G column which enables efficient separation of the IgG fraction. Following IgG elution, the buffer was replaced by PBS, and the concentration of purified IgG was measured with a spectrophotometer.

Results

Passive Sensitization Using the Patients' Sera

In the first series of experiments, the basophil-sensitizing capacity of the patients' pre- and post-SIT sera was analyzed. IgE-stripped basophils from nonallergic volunteers were passively sensitized with the patients' serum samples and then washed and stimulated with various concentrations of the cedar pollen extract. As shown in figure 1, after passive sensitization with the patients' sera, basophils obviously released histamine in response to the cedar pollen extract. In contrast, the cells without any passive sensitization step failed to respond to the pollen extract (data not shown). Importantly, the response curves after passive sensitization were practically indistinguishable between the pre- and post-SIT serum samples. These results clearly showed that the patients' pre- and post-SIT sera possessed essentially equivalent sensitizing capacity for human basophils.

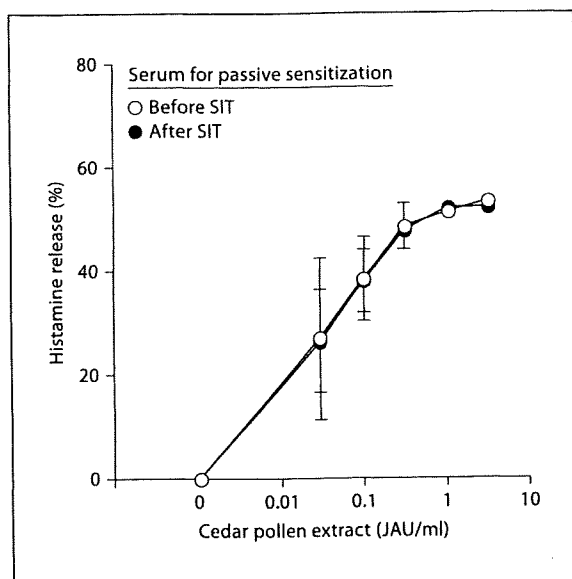


Fig. 1. Releasability of basophils passively sensitized with patients' serum samples. Basophils from nonallergic donors were semipurified by Percoll centrifugation, treated with IgE-stripping buffer and passively sensitized at 37°C for 2 h with patients' sera prepared before and after SIT. Cells were washed and stimulated with the indicated concentrations of Japanese cedar pollen extract, and histamine release was expressed as a percentage of the total cellular histamine after subtracting spontaneous release. Data are means \pm SEM of 3 separate experiments performed using serum samples from 3 different patients.

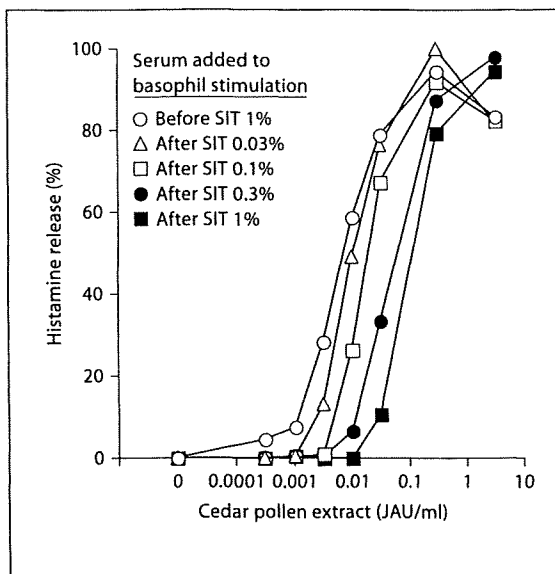


Fig. 2. Modification of cedar extract-induced histamine release by various concentrations of patient's serum. Diluted serum samples were mixed with cedar pollen extract and incubated for 30 min at 37°C before basophils obtained from a patient with pollinosis were added for histamine release reactions. Data are representative of 2 separate experiments, each showing similar results.

Histamine Release in the Presence of Sera Obtained before and after SIT: Demonstration of Blocking Antibody

Next, we evaluated the histamine release in the presence of the patients' pre- and post-SIT sera. Basophils were obtained from the peripheral blood of pollinosis subjects. After washing, basophils were stimulated with the cedar pollen extract that had been preincubated with diluted patients' serum samples. As shown in figure 2, cedar pollen extract-induced histamine release was obviously suppressed when the patients' post-SIT sera were added to the basophil-stimulating reaction medium to a final concentration of 0.03–1%, compared to addition of the pre-SIT sera. This histamine release blocking effect of the post-SIT serum samples was concentration dependent. Importantly, histamine release by a relatively low concentration of antigen was effectively blocked by the serum, although maximal histamine release by a high concentration of antigen was less affected. Based on these results, we decided to use serum samples at a 1% level of addition for analysis of histamine release modification.

The serum samples from all 5 patients were tested for their blocking effects on histamine release (fig. 3). For the sera from 3 patients (No. 2, 4 and 5), histamine release from passively sensitized basophils was strongly reduced by adding post-SIT serum samples, compared to pre-SIT sera. The blocking effect of the post-SIT sera on basophil histamine release was allergen specific, and not due to general inhibition of degranulation. The latter is clear, since there were no differences in histamine release induced by allergen nonspecific stimuli such as anti-Fc ϵ RI α -chain monoclonal antibody (CRA-1) and calcium ionophore A23187 when the effects of pre- and post-SIT sera were compared. The post-SIT sera from the 2 remaining patients (No. 1 and 3) showed no obvious blocking effects on histamine release: a slight decrease was observed only in the release evoked by threshold doses of cedar pollen extract (0.001 JAU/ml in fig. 3a and 0.00003 JAU/ml in fig. 3c). Note that patient No. 3 was a nonreleaser whose basophils failed to release histamine in response to IgE cross-linking stimulus.

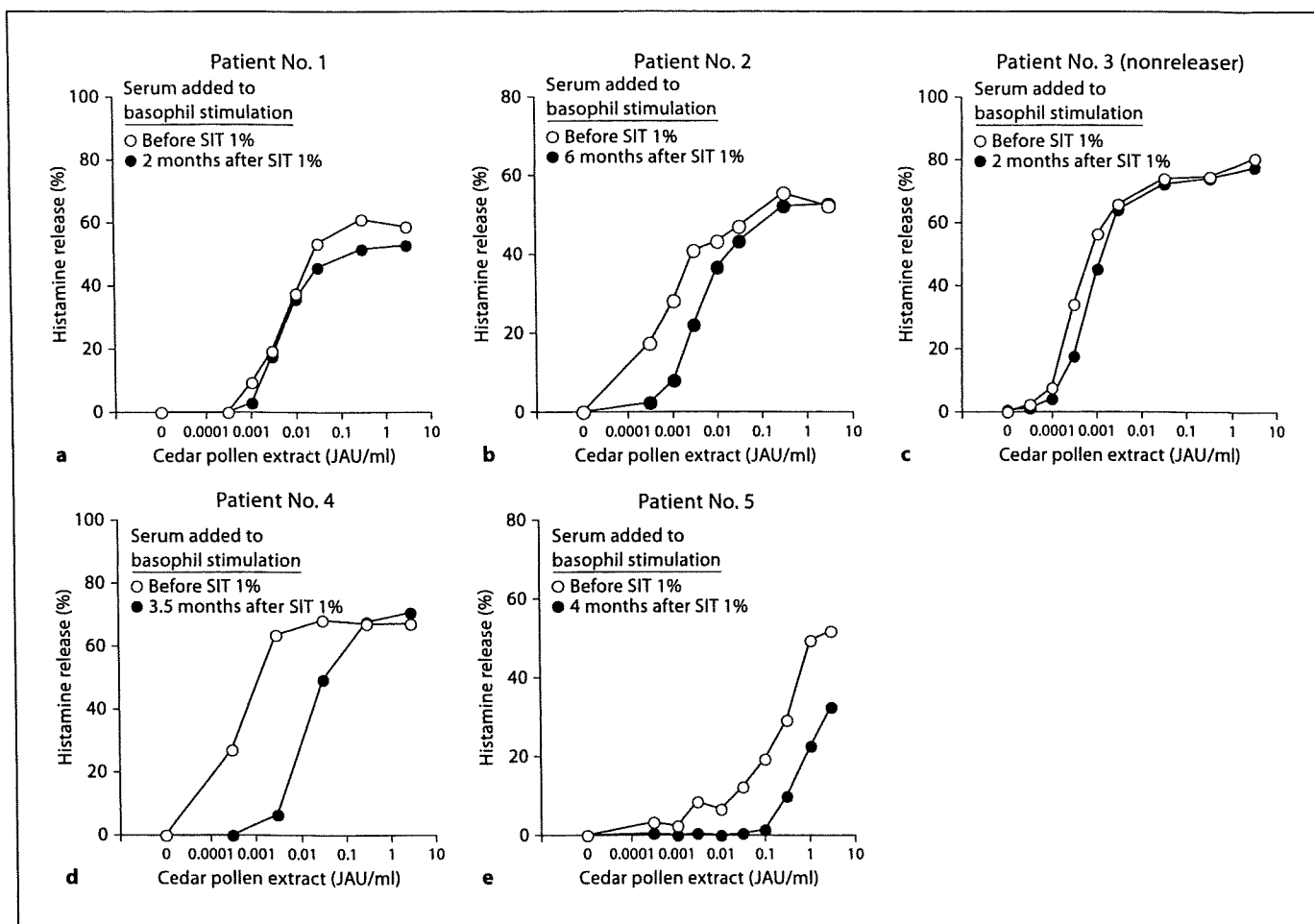


Fig. 3. Modification of cedar extract-induced histamine release by 5 patients' serum samples prepared before and after SIT. **a-e** Pre- and post-SIT sera from the 5 patients were mixed with cedar pollen extract and incubated for 30 min at 37°C before passively sensitized basophils were added. Each graph is representative of at

least 2 separate experiments showing similar results. One non-releaser whose basophils were unresponsive to IgE cross-linking stimulus was included in the 5 patients. **c** Graph of data from the nonreleaser's serum samples.

The results described above indicate that some, if not all, patients show obvious blocking activity in their sera after undergoing SIT. Next, we analyzed the blocking effect of sera prepared at several time points after SIT was initiated. As shown in figure 4, inhibition of low-dose antigen-induced histamine release was suppressed by sera prepared as early as 1.5 months after SIT was started. Histamine release blocking activity was stronger in sera obtained later. Another experiment using serum samples from another patient demonstrated somewhat different results: obvious blocking activity was detected in sera prepared 2 months after SIT, and serum samples taken at later times showed similar levels of blocking activity. These results show that apparent blocking activity is in-

duced relatively early (within 1 or 2 months) after the start of SIT.

The antigen-specific substance blocking histamine release may be reasonably surmised to be IgG, but specific IgE in the serum might also be involved in this blocking. We thus analyzed the blocking effect of serum samples prepared after SIT, with and without IgE-inactivating treatment by heating at 56°C for 2 h before performing the histamine release experiments. This heating procedure theoretically destroys the capacity of the Fc portion of IgE to bind to FcεRI expressed on basophils. As shown in figure 5, nonheated and heated post-SIT sera exhibited similar blocking activity on antigen-induced basophil histamine release. These results show that the blocking

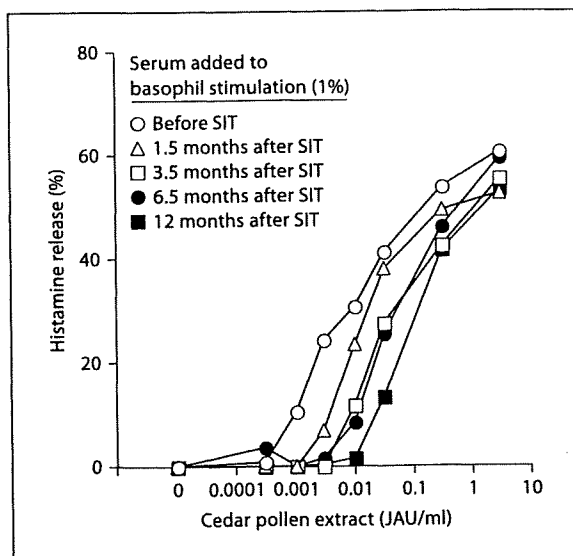


Fig. 4. Alteration of antigen-induced histamine release by serum samples obtained at various time points before and after SIT. Cedar pollen extract was mixed with the patient's serum and incubated for 30 min at 37°C before passively sensitized basophils were added. Data shown are derived from an experiment using the sera of patient No. 4.

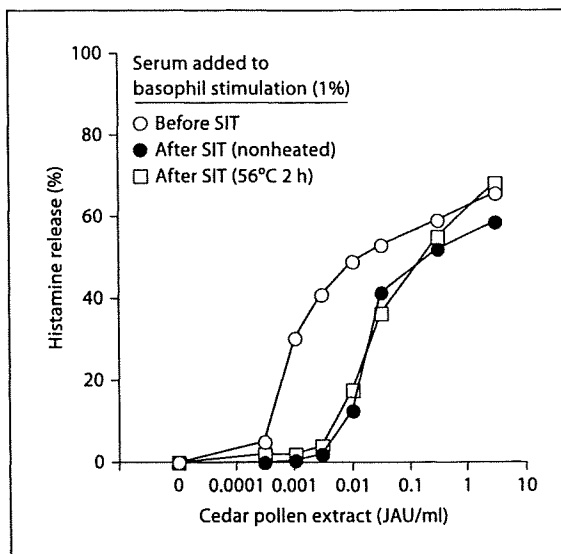


Fig. 5. Heat-resistant suppressive effect of post-SIT sera on antigen-induced histamine release. Cedar pollen extract was mixed with 3 serum samples from 1 patient: serum taken before SIT, serum taken after SIT (nonheated) and serum taken after SIT and heated for 2 h at 56°C before the histamine release experiment. After 30 min of incubation, passively sensitized basophils were added to conduct the histamine release reaction. Data shown are from an experiment using the sera from patient No. 5; another experiment generated similar results.

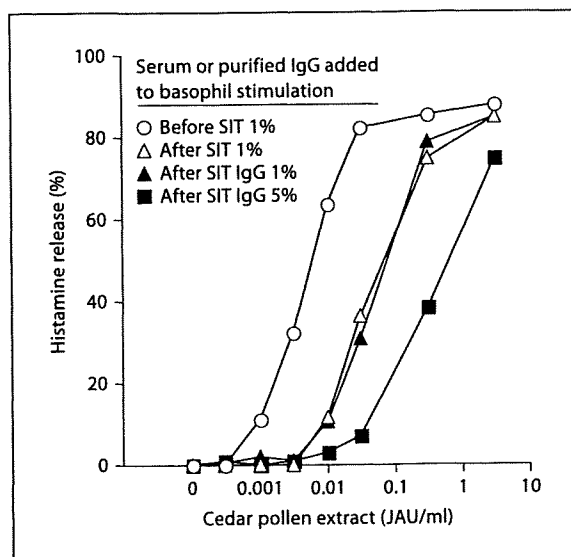


Fig. 6. IgG purified from post-SIT serum exhibited a suppressive effect on antigen-induced histamine release. Before stimulation of basophils obtained from a patient with pollinosis, cedar pollen extract was mixed with either pre- or post-SIT serum at 1% or purified IgG at 1 or 5% doses corresponding to post-SIT serum. Data are representatives of 2 experiments using the serum samples from patient No. 5; another experiment generated similar results.

potency found in the serum is heat resistant and is not mediated by the heat-sensitive Fc portion of IgE.

Next, we evaluated whether this blocking effect on histamine release was induced by the IgG fraction in serum. We purified IgG from post-SIT sera using a protein G column. The concentration of IgG was measured, and diluted IgG preparation was added to the basophil stimulation buffer to obtain a final IgG concentration corresponding to 1 or 5% of the patient's post-SIT serum. As shown in figure 6, the inhibitory effect on histamine release of purified IgG at a 1% concentration was essentially similar to that of 1% serum. In addition, IgG added to a higher concentration, that is 5%, showed a stronger inhibitory effect compared to IgG at 1%. Based on these results, the blocking activity included in post-SIT serum was thought to be due to IgG.

Discussion

In this study, we analyzed the induction of blocking antibody during SIT using a standardized allergen extract of Japanese cedar pollen. Patients' post-SIT sera po-

tently suppressed allergen-induced degranulation of sensitized basophils, and the suppressive effect was clearly observed for both basophils passively sensitized in vitro and cells obtained from patients with pollinosis (fig. 5, 6). This suppression was especially obvious when the allergen concentration was relatively low. The inhibitory effect of the post-SIT sera on basophil degranulation was allergen specific and unchanged even when the sera were preincubated at 56°C for 2 h. Purification experiments showed the protein G-separated IgG fraction accounts for the concentration-dependent blocking effect observed in post-SIT sera. These results indicate that the blocking antibody was IgG that was produced in the pollinosis patients during SIT.

The concept of blocking antibody was first introduced by Cooke et al. [9] and Loveless [10]. Approximately 40 years ago, Lichtenstein et al. [11] analyzed sera from allergic rhinitis patients who had undergone ragweed immunotherapy and proved that blocking antibody belongs to IgG, especially the IgG₄ subclass. Although it had not previously been demonstrated that SIT using the Japanese cedar pollen extract was associated with the production of specific blocking antibody, our present results clearly indicate that to be true. Use of a standardized allergen permits reliable assessment of the blocking efficacy of serum antibodies. In this study, we found that both the threshold dose of allergen extract required for stimulation of patients' basophils and the potency of the blocking antibody generated during SIT varied widely among our pollinosis patients, although the sample was small in number. Blocking antibody may partly account for the clinical efficacy of SIT and also for the paucity of systemic adverse events during allergen administration. Analysis of whether induction of blocking IgG antibody is really involved in the induction of the clinical efficacy of SIT may need to focus on the presence or absence of blocking antibody in SIT-ineffective subjects with this disease.

Recent progress in basic research has strongly suggested that the clinical efficacy of SIT derives from multiple mechanisms [2] and is not restricted to production of blocking antibody. It has been suggested that SIT corrects the imbalance between Th1 and Th2 lymphocytes, resulting in suppression of allergen-specific Th2 responses [12, 13]. It has also been demonstrated that functional activation of regulatory T cells may be involved in the SIT-induced suppression of Th2 responses [14–16]. These mechanisms may explain why SIT can reduce subsequent development of atopic diseases such as asthma [17–20]. In Japan, many people, including those who have never had atopic diseases and possess normal serum levels of total IgE, suffer from allergic rhinitis and conjunctivitis caused by Japanese cedar pollen [1]. In addition, more than half the population has been demonstrated to be sensitized to this pollen, possessing serum IgE specific for it. Based on the strikingly high prevalence of Japanese cedar pollinosis and sensitization to the pollen, this pollinosis is believed to occur not only in atopic subjects but also in non-atopics. It will thus be important to clarify how pollinosis can develop in nonatopics and to analyze whether the multiple events known to be induced when SIT is administered for atopic asthma and rhinitis can also explain the clinical efficacy of SIT for Japanese cedar pollinosis.

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Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of the article.

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Age-Related Changes of Transforming Growth Factor β 1 in Japanese Children

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ABSTRACT

Background: Transforming growth factor β 1 (TGF β 1) is an important factor in immunomodulation. The expression of TGF β 1 has been shown to be influenced by the C-509T polymorphism in the TGF β 1 gene. We investigated age-related changes of plasma TGF β 1 levels in a birth-cohort study. In addition, the genotypes of the C-509T polymorphism were investigated in allergic and non-allergic subjects.

Methods: Sixty-four neonates who met the following criteria were enrolled in this cohort study: 1) full-term vaginally delivery; 2) underwent DNA polymorphism analysis; and 3) questionnaire forms were filled out by parents at 0, 6 and 14 months of age. The umbilical cord blood at 0 months and peripheral blood at 6, and 14 months were collected. Plasma TGF β 1 levels were measured at 0, 6 and 14 months of age. Genomic DNA was extracted from their umbilical cord blood. The genotype of the subjects was examined for the presence of C-509T.

Results: The plasma TGF β 1 level at 6 months was the highest of the 3 measurements (at 0, 6, and 14 months of age). The TGF β 1 levels at 14 months in allergic subjects were significantly higher than those in non-allergic subjects ($p = 0.03$). All subjects with bronchial asthma ($n = 3$) had the TT genotype of the C-509T polymorphism.

Conclusions: The plasma TGF β 1 levels change with age. In addition, TGF β 1 may play a role in the pathogenesis of bronchial asthma.

KEY WORDS

bronchial asthma, single nucleotide polymorphism (SNP), transforming growth factor β 1 (TGF β 1), umbilical cord blood

INTRODUCTION

Transforming growth factor β 1 (TGF β 1) is a 25 kDa disulfide-linked homodimeric multifunctional cytokine. TGF β 1 may be the most important growth factor related to immunomodulatory effects because knockout mice died of massive inflammatory lesions.¹ The role of TGF β 1 in allergic diseases have been reported.²⁻⁴ The levels of TGF β 1 concentration were found to be higher in the bronchoalveolar lavage fluid of patients with asthma compared with subjects without asthma.³ The expression of TGF β 1 was found to be influenced by polymorphisms in the TGF β 1 gene, some of which may be associated with bronchial

asthma and other diseases.^{4,5} In particular, the T allele of the C-509T polymorphism within the TGF β 1 gene was associated with elevated serum TGF β 1 levels and also associated with elevated levels of total IgE in allergic asthma patients.^{5,6}

In this study, we investigated the age-related changes of TGF β 1 levels in a birth-cohort study. In addition, C-509T within the TGF β 1 gene in subjects with asthma was compared with non-allergic subjects.

METHODS

SUBJECTS

Sixty-four neonates born at Iwasa Maternity Hospital in Gifu Prefecture between November 2005 and July

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Table 1 Characteristics of subjects

Gender	Male	32	Female	32
Birth body weight (g)	Mean±SD	3137.1 ± 400.5 (range: 2410–4026g)		
Gestational week	Mean±SD	39w3d ± 1w2d (range: 37w6d–41w1d)		
Family history of allergy	Father	With allergy 25		
		Without allergy 32		
	Mother	With allergy 34		
		Without allergy 24		
No record		7		
Family smoking history with 1 or both parents still smoking	Present	16	Absent	48

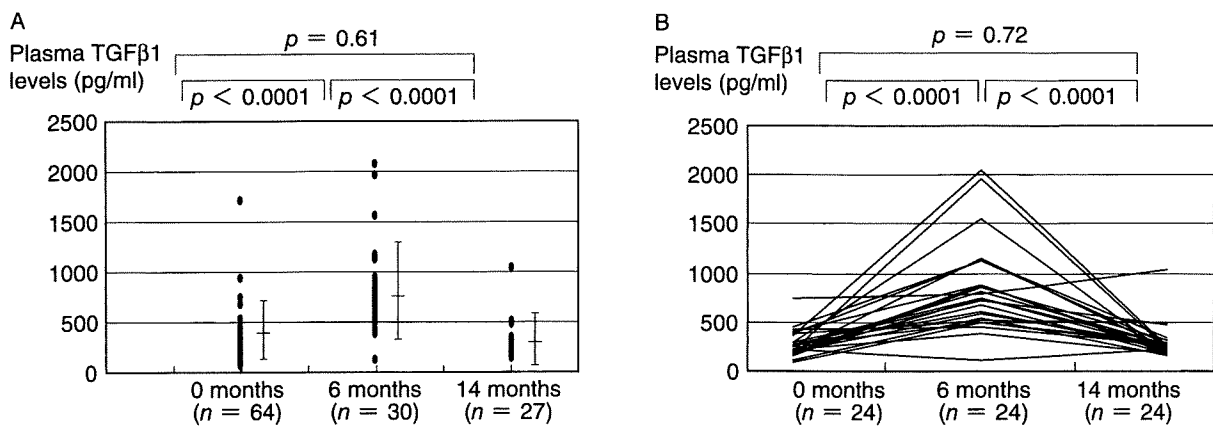


Fig. 1 (A) Plasma TGFβ1 levels at 0, 6, and 14 months of age. (B) Changes of plasma TGFβ1 levels in 24 subjects whose data were available at all three points.

2006, and satisfying the following criteria were enrolled in this cohort study: 1) full-term vaginally delivery; 2) underwent DNA polymorphism analysis; and 3) questionnaire forms were filled out by parents at 0, 6 and 14 months of age. Informed consent, including DNA analysis, was obtained from the parents of the neonates during their stay in the hospital. This cohort study was approved by the Ethical Committee of the Graduate School of Medicine of Gifu University. Umbilical cord blood samples were collected from 64 neonates and peripheral blood samples were also collected for analysis from 30 subjects at 6 months and from 27 subjects at 14 months of age. Subjects who were given a diagnosis of atopic dermatitis and bronchial asthma during the follow-up over a period of 14 months after birth, were classified as having those diseases. Allergic subjects were defined as those who were given a diagnosis of atopic dermatitis and/or bronchial asthma and non-allergic subjects were defined as those having neither atopic dermatitis nor bronchial asthma during the 14-month observation period. Some data of the recruited population are summarized in Table 1.

ASSAYS FOR PLASMA IgE LEVELS AND PLASMA TGFβ1 LEVELS

Plasma samples obtained from heparinized blood were kept at -30°C . Plasma IgE levels were determined by chemiluminescent enzyme immunoassay and plasma TGFβ1 levels were measured with a human ELISA kit (R & D Systems, Minneapolis, MN, USA); the detection range was 31.2–2000 pg/ml.

DETECTION OF C-509T WITHIN THE TGFβ1 GENE

Genomic DNA was extracted from the heparinized umbilical cord blood using a Sepagene kit (Sanko Junyaku, Tokyo, Japan). The TGFβ1 gene was amplified and sequenced using the PCR technique and an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The primers used for amplification of the DNA fragments were the forward primer at position -737–-718 (5'-CAGACTCTAGAGACTGTCAG-3') and the reverse primer at position -320–-338 (5'-GTCACCAGAGAAAGAGGAC-3').

STATISTICAL ANALYSES

The relations between age and plasma in TGFβ1 lev-

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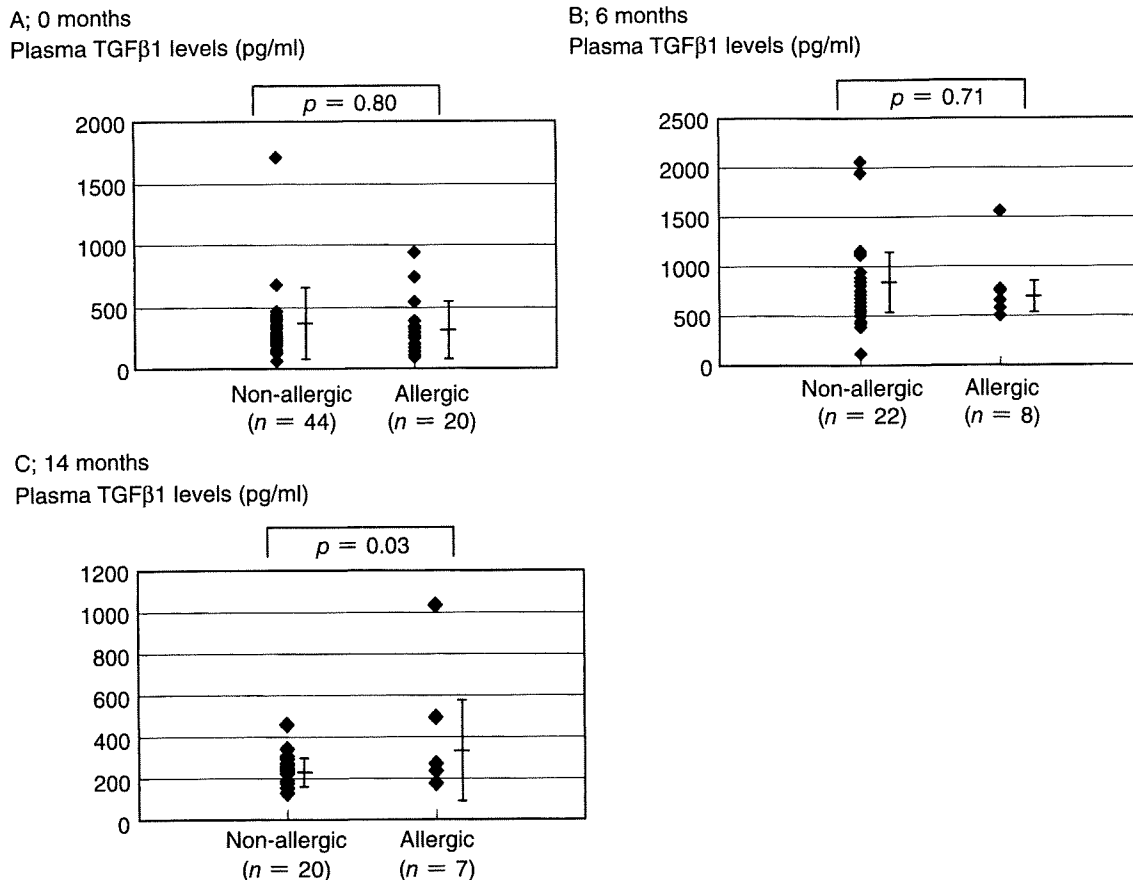


Fig. 2 Plasma TGF β 1 levels of non-allergic and allergic subjects at 0 months (A), 6 months (B) and 14 months (C) are shown. The plasma TGF β 1 levels at 14 months of age in allergic subjects were significantly higher than those in non-allergic subjects ($p = 0.03$, C).

els were determined by using the Mann-Whitney unpaired U-test. Pearson's correlation coefficient was used for statistical analysis of plasma IgE levels and TGF β 1 levels. The chi-square test was performed for statistical analysis of allergic symptoms and C-509T within the TGF β 1 gene. Probability (p) values of less than 0.05 were considered to indicate statistical significance.

RESULTS

ALLERGIC DISORDERS AND SYMPTOMS IN SUBJECTS

By the age of 14 months, 20 of the 64 subjects were allergic and 44 were non-allergic as defined in the Methods. Of the 64 subjects, 19 had atopic dermatitis and 3 had bronchial asthma, while two subjects suffered from both atopic dermatitis and bronchial asthma. Peripheral blood samples were collected at 6 months of age in 30 subjects and at 14 months of age in 27 subjects.

CHANGES IN PLASMA TGF β 1 LEVELS BY AGE

Plasma TGF β 1 levels at 0, 6, and 14 months of age

are shown in Figure 1A. Plasma TGF β 1 levels were 306.2 ± 230.9 pg/ml (M \pm SD) at 0 months ($n = 64$), 805.3 ± 428.4 pg/ml at 6 months ($n = 30$), and 273.6 ± 175.8 pg/ml at 14 months ($n = 27$). Plasma TGF β 1 levels at 6 months of age were significantly ($p < 0.0001$ for each) higher than those in both 0 months and 14 months.

TGF β 1 levels were measured at all 3 points in all 24 subjects (data shown in Fig. 1B). The plasma TGF β 1 levels at 6 months of age were significantly ($p < 0.0001$ for each) higher than at both 0 and 14 months. This pattern was observed in 22 of the 24 subjects whose plasma TGF β 1 levels were measured at all 3 points.

RELATIONSHIP BETWEEN PLASMA TGF β 1 LEVELS AND ALLERGIC DISEASES OR PLASMA IgE LEVELS

Figure 2A, B, C show plasma TGF β 1 levels at 0, 6, and 14 months of age in non-allergic and allergic subjects. The plasma TGF β 1 levels at 14 months of age in allergic subjects were significantly higher than those in the non-allergic subjects ($p = 0.03$, Fig. 2C).

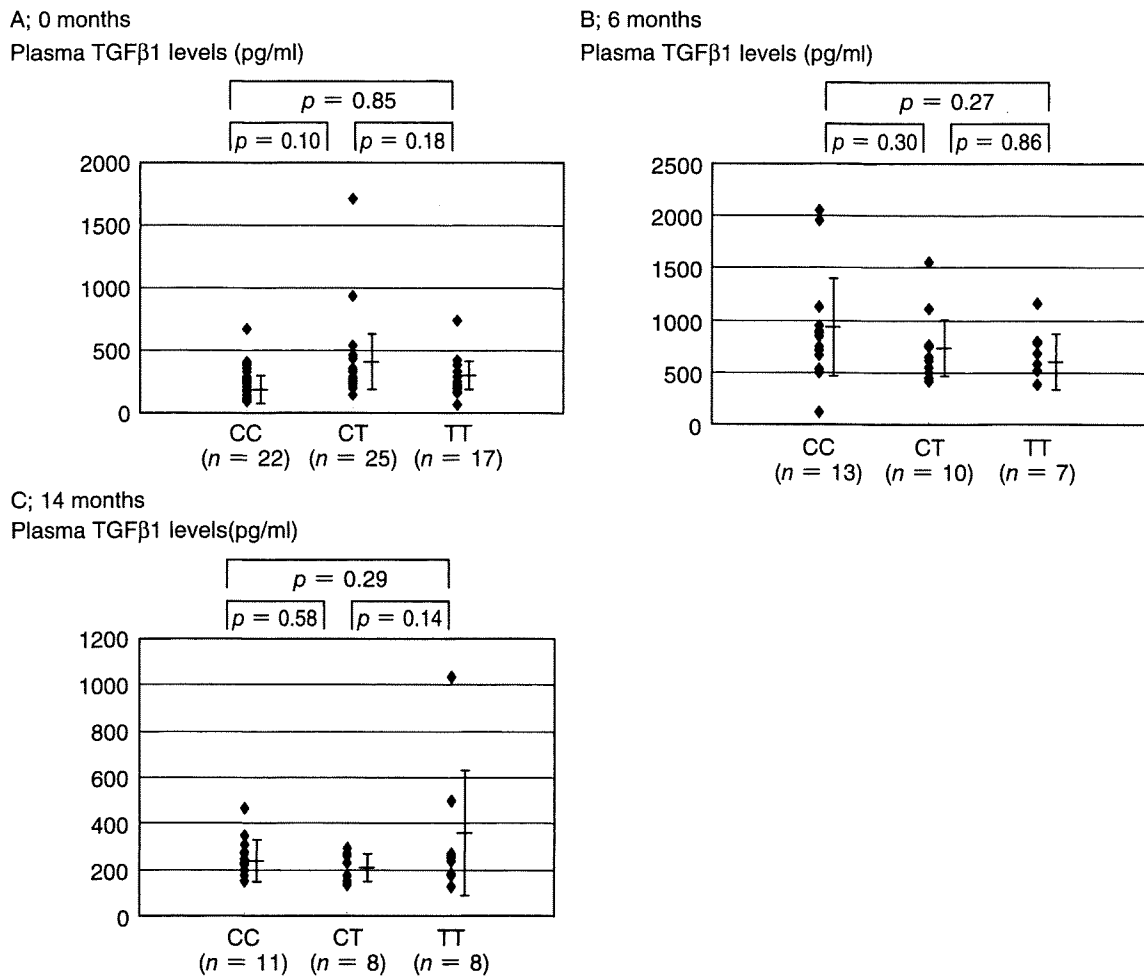


Fig. 3 Relationship between C-509T SNP of TGFβ1 gene and plasma TGFβ1 levels at 0 months (A), 6 months (B) and 14 months (C) is shown.

We also investigated the relationship between plasma IgE levels and TGFβ1 levels at 0, 6 and 14 months of age and found no significant relations (0 months; $p = 0.368$, 6 months; $p = 0.778$, 14 months; $p = 0.818$, data not shown).

PLASMA TGFβ1 LEVELS AND TGFβ1 C-509T POLYMORPHISM

Among the 64 subjects, 22, 25 and 17 were classified as genotype CC, CT, and TT, respectively. As shown in Figure 3, these genotypes were not significantly related with plasma TGFβ1 levels at either 0, 6, or 14 months of age.

TGFβ1 C-509T POLYMORPHISM AND ALLERGIC DISEASES

The genotypes of TGFβ1 C-509T polymorphism were not associated with the prevalence of atopic dermatitis at 6 months of age (Table 2) and 14 months of age (Table 3). However, the TT genotype was present in the TGFβ1 gene (C-509T) in all 3 patients with

asthma at 14 months of age (Table 4). Furthermore, the TGFβ1 levels ($M \pm SD$; 651.42 ± 544.79 pg/ml) in patients with asthma having the TT genotype in the TGFβ1 gene (C-509T) were higher than the TGFβ1 levels ($M \pm SD$; 243.75 ± 89.19 pg/ml) in subjects without asthma (CC, CT or TT genotypes) at 14 months.

DISCUSSION

TGFβ1 is a multifunctional cytokine that has immunomodulatory effects produced by airway epithelial cells, eosinophils, helper T type 2 lymphocytes, macrophages, and fibroblasts. An important finding in this study is the changing pattern of plasma TGFβ1 levels over time at 0, 6, and 14 months of age. Plasma TGFβ1 levels at 6 months of age were the highest among these 3 measured age points. This pattern was observed in 22 of the 24 subjects whose plasma TGFβ1 levels were available at all 3 points. Statistically, the plasma TGFβ1 level at 6 months of age was significantly higher than that at 0 months and at 14

Age-Related Changes of Transforming Growth Factor β 1

Table 2 Genotype frequency of C-509T *TGF- β 1* promoter SNP in subjects with/without atopic dermatitis at 6 months

	CC	CT	TT	Total
Without atopic dermatitis	18	17	12	47 (74%)
With atopic dermatitis	4	8	5	17 (26%)

$(p = 0.551)$

Table 3 Genotype frequency of C-509T *TGF- β 1* promoter SNP in subjects with/without atopic dermatitis at 14 months

	CC	CT	TT	Total
Without atopic dermatitis	18	19	12	49 (77%)
With atopic dermatitis	4	6	5	15 (23%)

$(p = 0.865)$

months of age ($p < 0.0001$ for each). We believe this is the first time this unique change in the plasma TGF β 1 level has been reported.

A previous study showed that the range of plasma TGF β 1 levels was 2000–4000 pg/ml in adult population.⁵ In our study, the range of plasma TGF β 1 levels at 0, 6, and 14 months of age was 0–2500 pg/ml, and was lower than that of adult populations. At present, we do not know what causes the plasma TGF β 1 level changes, but one possible explanation could be that TGF β 1 is necessary for dramatic changes in immunological response or maturation at around 6 months of age. In other studies, the CD4 or CD8 cells producing cytokines, including TGF β 1, increased with age.^{7,8} An increase in the number of TGF β 1-producing cells may partially explain why TGF β 1 production at 6 months was higher than that at 0 months. We are planning to observe further changes in plasma TGF β 1 levels from 14 months of age to 5 years of age or older.

We could not find any association of IgE with TGF β 1 in this study. TGF β 1 is produced by various cells including airway epithelial cells, eosinophils, lymphocytes, macrophages, and fibroblasts.^{9,10} Various cell factors should be considered to evaluate the level of plasma TGF β 1.

It has been hypothesized that the T allele of the C-509T SNP enhances the Yin Yang 1 (YY1) transcription factor consensus binding site (-CCATCTC/TG-) on the TGF β 1 promoter and is responsible for increased TGF β 1 transcription.¹¹ In the present study there were no significant differences in plasma TGF β 1 levels at 0, 6, and 14 months of age regarding genotypes of *TGF β 1* C-509T. Pulleyn has shown that the T allele of the C-509T SNP is associated with the diagnosis of asthma and asthma severity.¹² In this study, only 3 subjects were given a diagnosis of bronchial asthma by the age of 14 months. Interestingly, the TT genotype was present in these 3 subjects (Table 4, $p = 0.016$). Although the prevalence of bron-

Table 4 Genotype frequency of C-509T *TGF- β 1* promoter SNP in subjects with/without bronchial asthma at 14 months

	CC	CT	TT	Total
Without bronchial asthma	22	25	14	61 (96%)
With bronchial asthma	0	0	3	3 (4%)

$(p = 0.016)$

chial asthma at 14 months of age is low we need to obtain data at 5 years of age for an accurate evaluation of the role of the *TGF β 1* polymorphism in bronchial asthma. We are planning to increase the number of subjects to participate in future studies and to conduct a follow up for a longer time span.

In conclusion, this birth-cohort study suggests that plasma TGF β 1 levels are influenced by age and that the C-509T SNP of the TGF β 1 gene is an important susceptibility locus for asthma in infants at 14 months of age, despite the fact that the number of subjects who participated in this study was limited.

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