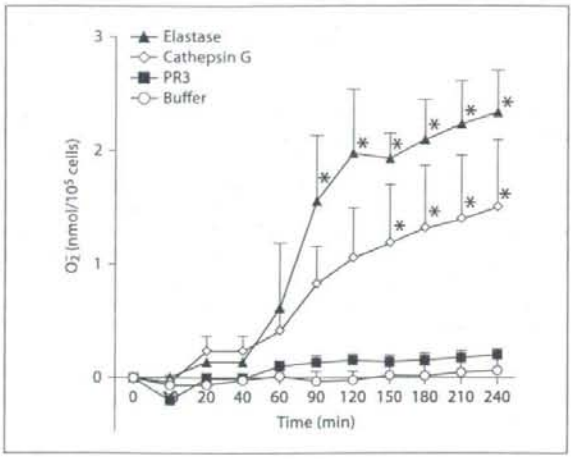
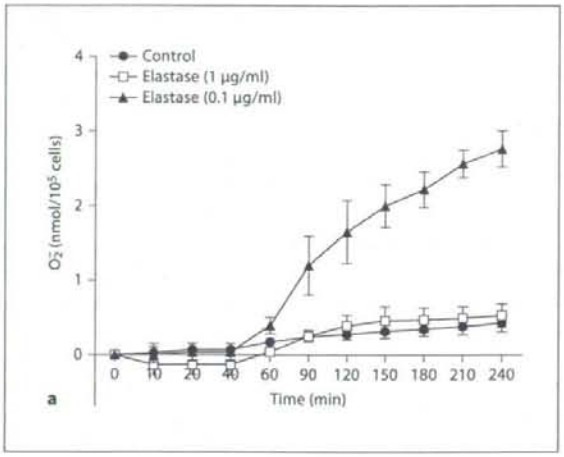


Fig. 1. Generation of O_2^- anion by eosinophils incubated for 4 h with neutrophil proteases at 1 μg/ml (n = 3). * $p < 0.05$ vs. buffer control.



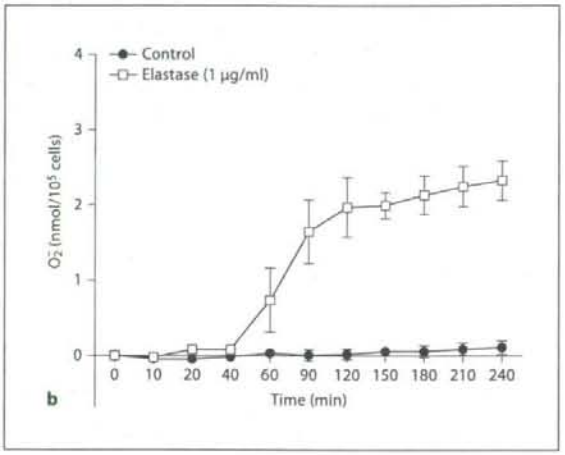


Fig. 2. Generation of O_2^- anion by eosinophils with elastase from different donors. **a** Highest responses at a concentration of 0.1 μg/ml (n = 3). **b** Highest responses at a concentration of 1 μg/ml (n = 3).

cathepsin G and that O_2^- production with elastase was higher than that with cathepsin G, although the difference was not statistically significant. We also examined concentration-dependent effect of elastase and found that optimal concentration of elastase in inducing superoxide generation was variable among donors (fig. 2), namely at 1 μg/ml or 0.1 μg/ml. Based on these results, the latter experiments were performed with elastase at a concentration that gave an optimal stimulation for a donor.

We then studied the inhibitory effect of sivelestat sodium hydrate, a selective neutrophil elastase inhibitor [13]. Sivelestat significantly inhibited elastase-induced O_2^- generation (fig. 3a). PMSF, a serine protease inhibitor, also significantly, but partially, suppressed O_2^- generation by elastase (fig. 3b). Because an organic solvent for PMSF, N,N-dimethylformamide (Sigma), showed a toxic effect for eosinophils at higher concentrations, the concentration of PMSF employed in this experiment was the highest possible to prevent the nonspecific suppressive effects of the solvent and we could not test higher concentrations usually used for protease inhibition for other cell types.

Calcium Influx

Elastase induced increase in intracellular Ca^{2+} of stimulated eosinophils (fig. 4).

Cytokine and Chemokine Production

Neutrophil elastase, cathepsin G and PR3 induced production of IL-6, TNF-α, IL-8 and GRO-α (fig. 5). Among a panel of cytokines and chemokines tested, production of IL-1β, IL-2, IL-4, IL-5, IL-10, IL-13, IFN-γ, GM-CSF, MIP-1α, MIP-1β, MCP-1, MCP-3, RANTES and eotaxin was not evident. Potency of each protease in inducing the cytokines and chemokines was somewhat different but it is of note that PR3, which had no effect in inducing superoxide generation, induced significant production of the cytokines/chemokines.

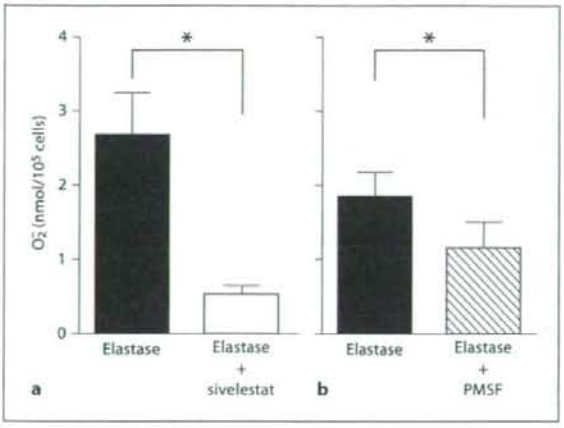


Fig. 3. Inhibition of O_2^- anion generation with elastase by sivelestat sodium hydrate (**a**) and by PMSF (**b**). The results show measured value at 120 min of incubation (n = 4). * $p < 0.05$.

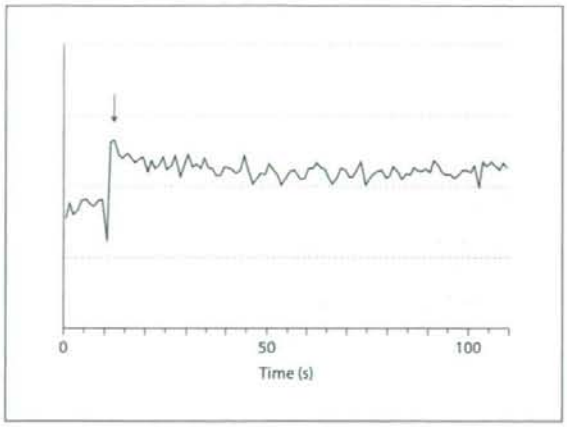


Fig. 4. Calcium influx to eosinophils induced by elastase. Eosinophils were stimulated with neutrophil elastase at a concentration of 1 μg/ml. The data shown are representative of 2 independent analyses from different donors, each showing similar results. The arrow indicates the addition of elastase.

Discussion

In the present study, we have demonstrated that neutrophil serine proteases activated eosinophils in vitro to cause superoxide generation and cytokine/chemokine secretion. To our knowledge, this is the first observation that neutrophil proteases directly enhance eosinophil effector functions. Especially, it is of note that the cytokines and chemokines produced by eosinophils were well-known proinflammatory and neutrophil-chemotactic molecules, namely IL-6, TNF-α, IL-8 and GRO-α.

Accumulation and activation of neutrophils in the airways has been reported in severe refractory asthma in adults [1, 2, 14] as well as acute asthma exacerbation in young children [15, 16]. Neutrophils may aggravate airway inflammation in asthma where eosinophils are presumably major effector cells [17]. Kikuchi et al. [4] reported that the percentage of eosinophils in induced sputum from patients with asthma was significantly higher in those with airway neutrophilia and that the percentage of neutrophils was significantly correlated with the percentage of eosinophils in the sputum from severe asthma patients. A report from ENFUMOSA also demonstrated colocalization as well as coactivation of neutrophils and eosinophils in the airways of severe asthma patients [14]. Collectively, it is suggested that neutrophilic inflammation enhances eosinophilic inflammation in severe asthma.

Exploration for the mechanism by which neutrophils enhance eosinophil accumulation and activation in asthma, however, has just begun to take shape. Nagata and his group recently demonstrated with their elegant TBM model that when eosinophils were co-incubated with neutrophils and stimulated with IL-8, the TBM of eosinophils was significantly augmented apparently due to several mediators secreted from activated and transmigrated neutrophils, including leukotriene B4, platelet-activating factor, TNF-α and matrix metalloproteinase 9 [5]. Our observation that neutrophil-derived proteases enhanced superoxide generation and proinflammatory cytokine/chemokine production from eosinophils may add another mechanism for the theoretical interaction of the 2 cell types in severe asthma.

Eosinophils secrete a variety of cytokines and chemokines [18]. They have potentials to promote allergic inflammation by producing Th2-type cytokines such as IL-4 [19], IL-13 [20] and IL-9 [9] or to cause airway remodeling by producing TGF-β [21, 22]. Although precise mechanisms for the differential production of cytokines by eosinophils are not well known, the cells may respond to different stimuli in different microenvironments, resulting in differential expression of cytokines. We found that neutrophil proteases induced secretion of neutrophilic chemokines, IL-8 and GRO-α, suggesting the presence of a positive feedback mechanism for neutrophil re-

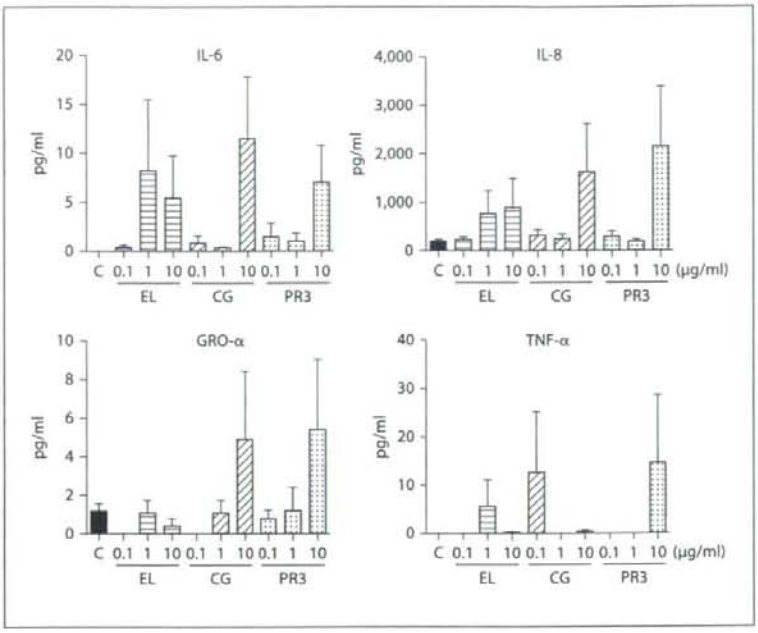


Fig. 5. Cytokine and chemokine production from eosinophils induced by neutrophil proteases. Eosinophils were stimulated for 48 h with elastase (EL), cathepsin G (CG) and PR3 at the concentrations indicated (n = 4). C = Control.

cruitment. We also observed TNF-α and IL-6 production, which are implicated in Th17-driven neutrophilic inflammation in asthma [23–26].

We recognize some shortcomings in the study. First, although the proteases used in the present study were well-known serine proteases, we did not demonstrate direct evidence for PAR-2 dependency of the induced eosinophil functions. Because the serine protease inhibitor PMSF at higher concentrations commonly employed for other cell types was toxic to eosinophils (not toxic at lower concentrations), we were not able to use it at sufficient concentrations for complete inhibition, merely resulting in partial inhibition (fig. 3b). In addition, an interesting observation that PR3 induced cytokine production without inducing superoxide should also be addressed for the mechanism. Each serine protease from neutrophils may recognize different sites of the receptor molecule, which then induce different functions, and further study needs to be done. Second, dose dependency in cytokine-inducing activities of elastase, cathepsin G and PR3 was not clearly seen, although IL-8 production by elastase and GRO-α production by cathepsin G and PR3 appeared to be dose dependent. We assume that the discrepancy may be attributed to the fact that optimal concentrations of the proteases for inducing cytokine production may be variable among donors.

In conclusion, we suggest the possibility that neutrophil proteases may enhance airway inflammation in asthma through activation of eosinophils to produce superoxide and neutrophilic cytokines and chemokines. The mechanism may underlie a part of the pathogenesis of severe asthma and effective inhibition of the proteases can be a future therapeutic target.

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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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Original Paper

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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 · IgG_4 · 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 FcεRIα 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 IgG_4 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 IgG_4 titers were significantly elevated at 1 month and remained at high levels 12 months after the treatment. Stimulation 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.

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 μg/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 IgG_4

Each well of a 96-well microplate (Costar, Corning, N.Y., USA) was filled with 100 μl of JCP extract (Torii) at 100 μg/ml in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3.1 mM NaN_3, 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 IgG_4 (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 IgG_4 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].

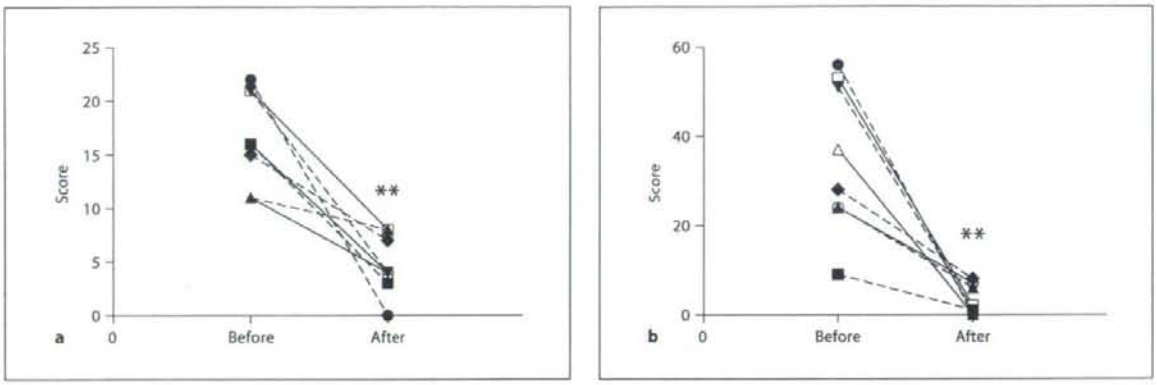

Fig. 1. Changes in symptom score (NSS; **a**) and quality of life score (QOLD; **b**) before and after RIT. Dotted lines represent patients who experienced the pollen season in 2005 before RIT and in 2006 after RIT. Solid lines represent patients who experienced the pollen season in 2006 before RIT and 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/mm^3 in 2005, 2,643/mm^3 in 2006 and 3,144/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, IgG$_4$, HRT and CD203c Expression before and after RIT

There were no statistical changes in JCP-specific IgE levels after RIT (fig. 2a). In contrast, IgG$_4$ 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 μg/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.

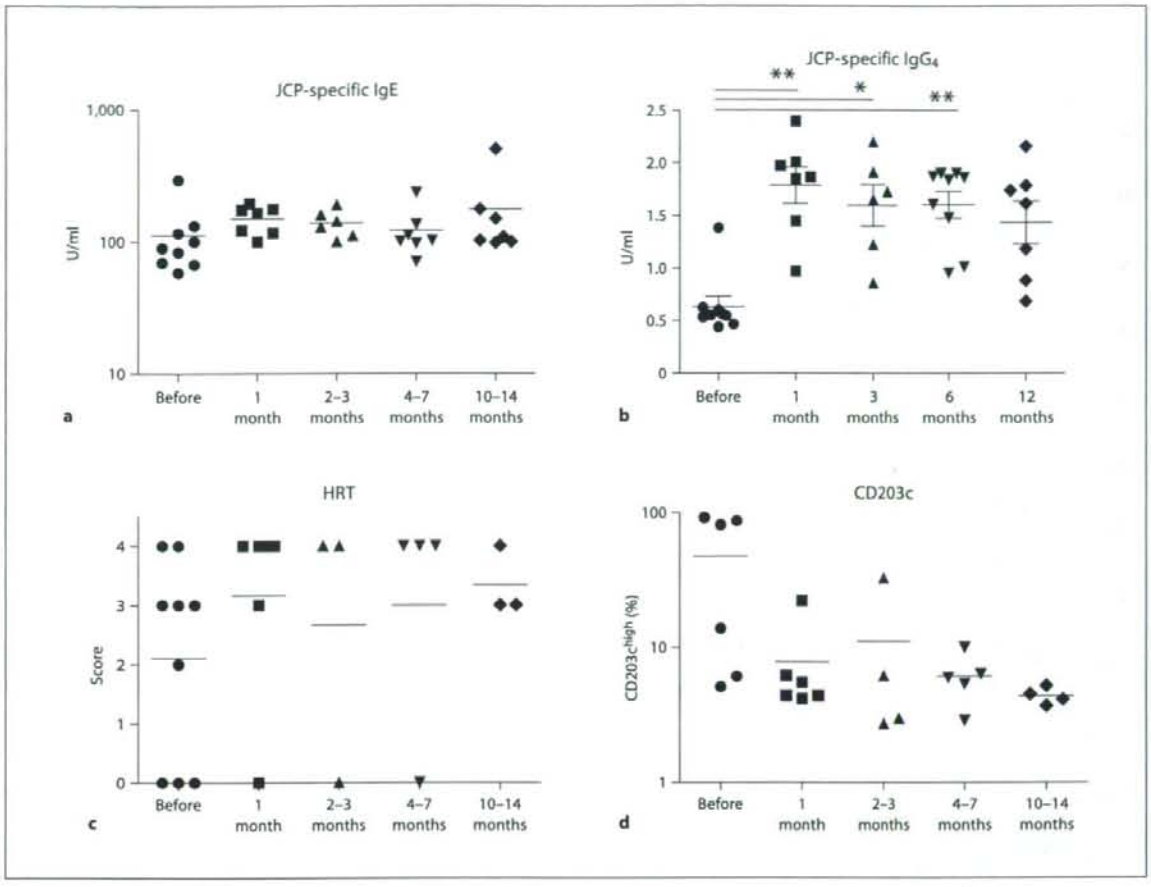

Fig. 2. Changes in JCP-specific IgE levels (**a**), JCP-specific IgG_4 levels (**b**), JCP-induced basophil histamine release score in HRT (**c**) and JCP-induced percentage of $CD203c^{high}$ in basophils (**d**). * $p < 0.05$, ** $p < 0.01$, Dunn's multiple comparison test.

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

	RCE (n = 4)		NRCE (n = 3)		Nonresponders (n = 2)	
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%)	
	before	after	before	after	before	after
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.

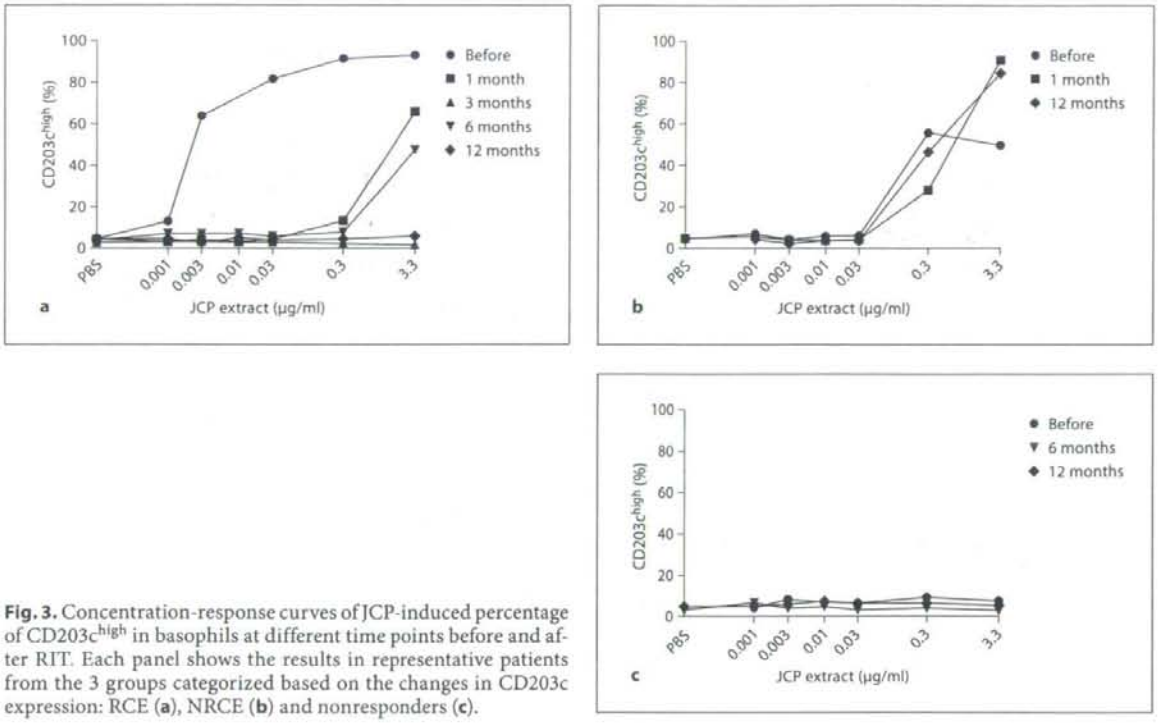

Fig. 3. Concentration-response curves of JCP-induced percentage of $CD203c^{high}$ in basophils at different time points before and after RIT. Each panel shows the results in representative patients from the 3 groups categorized based on the changes in CD203c expression: RCE (**a**), NRCE (**b**) and 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 IgG_4 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, IgG_1 rises first, followed by IgG_4 that remains at high levels, whereas IgG_1 starts to decline after some time [22–24]. Our data, demonstrating significant increase in IgG_4 levels after RIT in JCP pollinosis, confirmed the previous observations in other allergen-specific immunotherapies. 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 IgG_4 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 IgG_4 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 IgG_4 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 IgG_4 production [27]. In this context, the elevation of JCP-specific IgG_4 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. ***Conclusions:*** 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.

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 IgG_4 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.

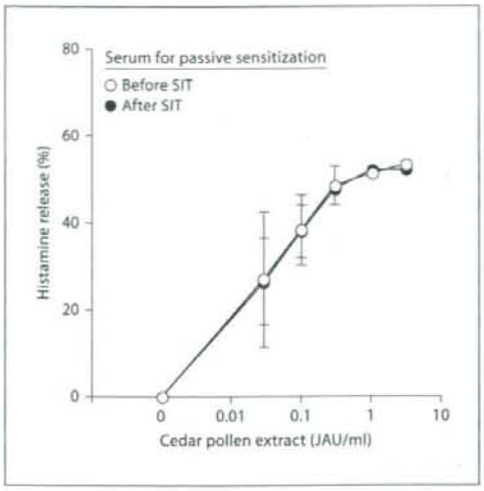

Fig. 1. Releasability of basophils passively sensitized with patients' serum samples. Basophils from nonallergic donors were semipurified by Percoll centrifugation, treated with IgE-stripping buffer and passively sensitized at 37°C for 2 h with patients' sera prepared before and after SIT. Cells were 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 ± SEM of 3 separate experiments performed using serum samples from 3 different patients.

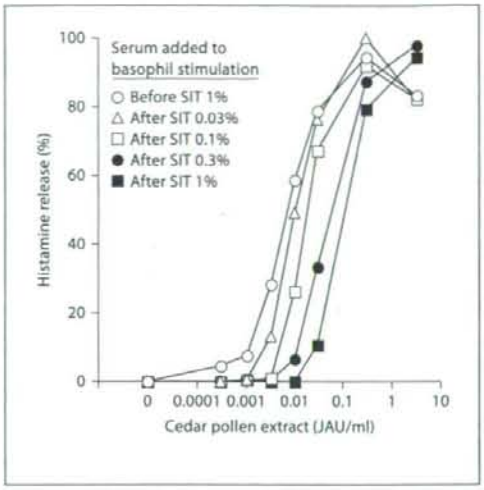

Fig. 2. Modification of cedar extract-induced histamine release by various concentrations of patient's serum. Diluted serum samples were mixed with cedar pollen extract and incubated for 30 min at 37°C before basophils obtained from a patient with pollinosis were added for histamine release reactions. 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-FcεRI 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.

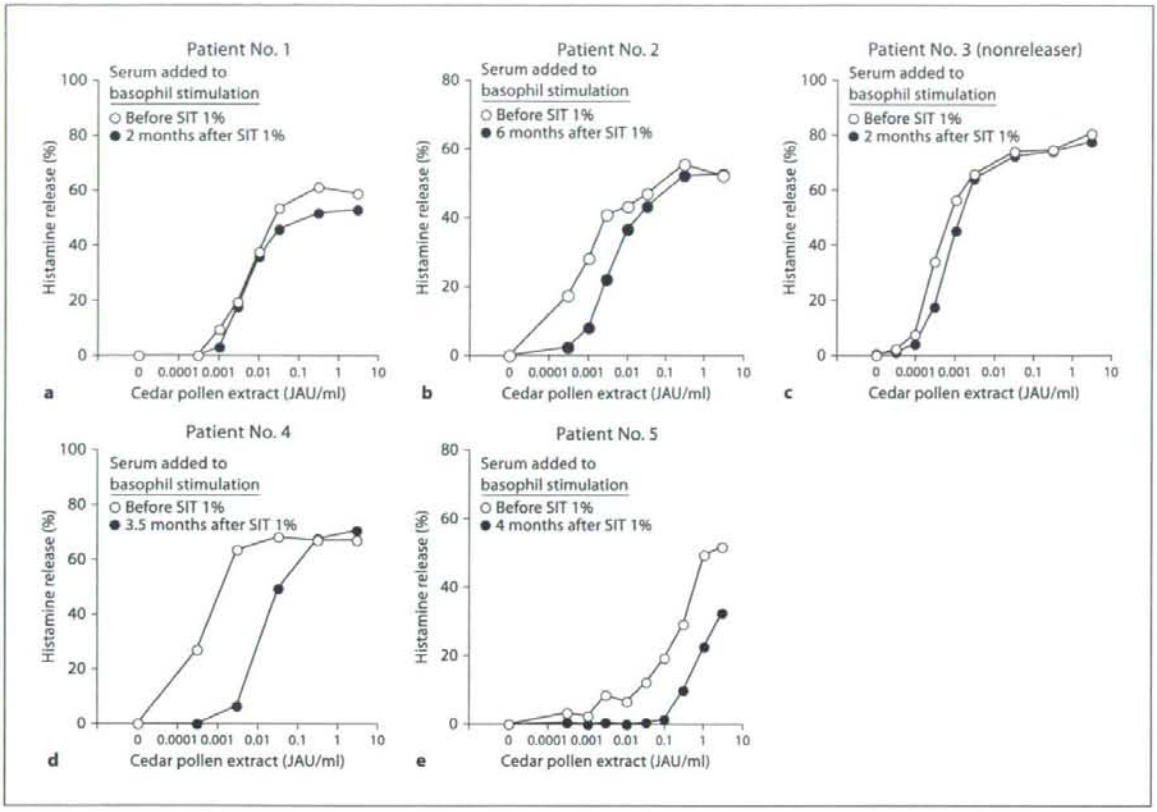

Fig. 3. Modification of cedar extract-induced histamine release by 5 patients' serum samples prepared before and after SIT. **a–e** Pre- and post-SIT sera from the 5 patients were mixed with cedar pollen extract and incubated for 30 min at 37°C before passively sensitized basophils were added. Each graph is representative of at least 2 separate experiments showing similar results. One nonreleaser 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 induced 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

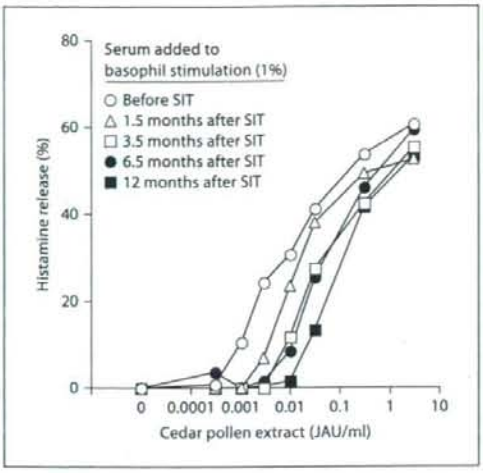

Fig. 4. Alteration of antigen-induced histamine release by serum samples obtained at various time points before and after SIT. Cedar pollen extract was mixed with the patient's serum and incubated for 30 min at 37°C before passively sensitized basophils were added. Data shown are derived from an experiment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

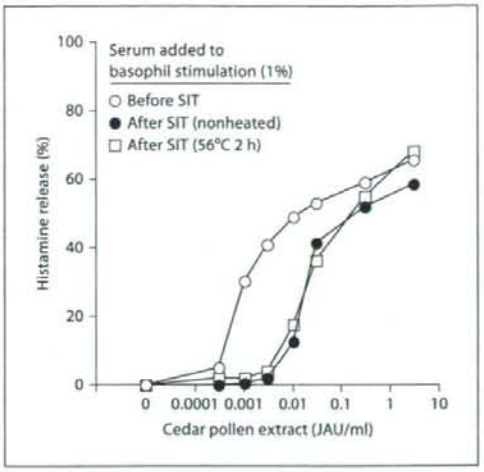

Fig. 5. Heat-resistant suppressive effect of post-SIT sera on antigen-induced histamine release. Cedar pollen extract was mixed with 3 serum samples from 1 patient: serum taken before SIT, serum taken after SIT (nonheated) and serum taken after SIT and heated for 2 h at 56°C before the histamine release 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.

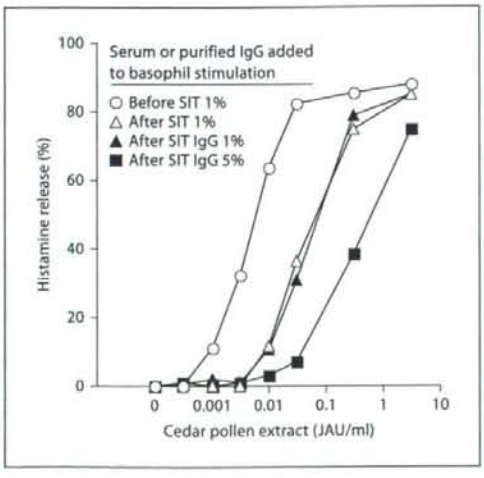

Fig. 6. IgG purified from post-SIT serum exhibited a suppressive effect on antigen-induced histamine release. Before stimulation of basophils obtained from a patient with pollinosis, cedar pollen extract was mixed with either pre- or post-SIT serum at 1% or purified IgG at 1 or 5% doses corresponding 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 IgG_4 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 nonatopics. 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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