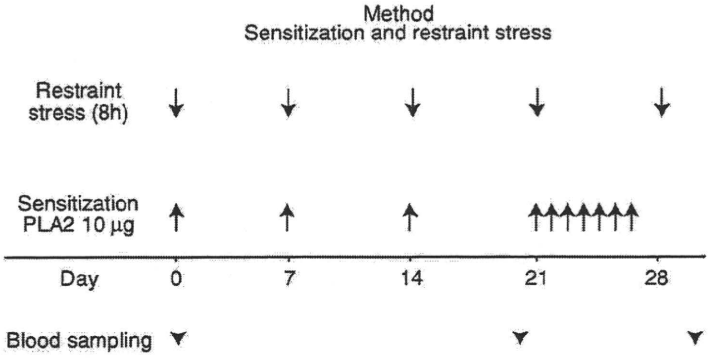

Fig. 1 Treatment schedule; Mice were intranasally sensitized to 10 μg of PLA2 in 20 μl saline. Sensitization was repeated in the same manner. Following sensitization, restraint stress was applied to mice using a single transparent cylindrical chamber and repeated once every week, for a total of 5 applications. Blood samples were taken from each tail vein at 0, 20, and 30 days after primary sensitization.

ever, little is known whether aging affects stress-induced alterations in humoral immune responses.

In this study, we compared stress-induced inhibitions of antibody production between aged and young mice in an intranasal sensitization model. As physical restraint is occasionally used in geriatric care in order to prevent bed fall in hospitals,[13] the results presented here may provide a basis for evaluating the risk of restraint stress on humoral immunity in elderly patients.

METHODS

ANIMALS

Nine-week old female, young adult mice (18–20 g) and 17- month old female, CBA/J strain mice (26–30 g) (Charles River Japan, Yokohama, Kanagawa, Japan) were used in this study. Mice were maintained in an animal house according to the guidelines of the Animal Study Committee of the Kagawa Prefectural College of Health Sciences. All animals were housed in groups of 3, each in an opaque polycarbonate mouse cage (30 × 20 × 30 cm) with access to food and water ad libitum, and were maintained on a 12-hour light-dark cycle for 2–3 weeks before the experiments began. The temperature in the animal house was maintained at 25℃.

REAGENTS

ELISA plates were purchased from Corning (Corning, NY, USA). Purified rat anti-mouse IgE was purchased from Biosource (Camarillo, CA, USA), extraAvidin-peroxidase conjugate, PLA2, carbonate buffer and fetal calf serum from Sigma (St. Louis, MO, USA), tetramethylbenzidine substrate from Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA), phospholic acid from Wako Pure Chemical Industries (Osaka, Japan), peroxidase-conjugated goat anti-mouse IgG1/IgG2a monoclonal antibody from Boehringer-Mannheim (Indianapolis, IN, USA) and biotin (long-arm) N-hydroxy succinimide ester from Vector Laboratories (Burlingame, CA, USA). It is known that endotoxin contamination suppresses allergen-induced immunologic responses including IgE Production on mice.[14] Contamination of endotoxin was negligible as determined using an Endospec assay kit (Seikagaku Kogyo, Tokyo, Japan) in accordance with the manufacturer's instructions.

SENSITIZATION OF MICE

Mice (n = 6–8 per group) were sensitized by nasal administration of 20 μl of saline containing 10 μg of PLA2 using a microsyringe (Hamilton, Reno, NV, USA). PLA2 was carefully given as 7–8 drops of aqueous solution into each nostril in turn. Sensitization was repeated in the same manner after 1 and 2 weeks. On day 21 and on the following 7 consecutive days, the same amount of PLA2 was given in the same manner. Blood samples were taken from the tail vein on days 0, 20, and 30 after primary sensitization (Fig. 1).

INDUCTION FOR RESTRAINT STRESS

Following sensitization, restraint stress was applied to mice (n = 6–8 per group) using a single transparent polymethylmethacrylate cylindrical chamber (20 mm diameter, 100 mm long) commonly used for drawing blood from mice. This chamber was placed horizontally in the mouse cage, and the mice were maintained therein for a continuous 8-hour period without food or water. This manipulation was performed once a week, on a total of 5 occasions (Fig. 1). Control mice were maintained in their cages without food and water at the same time. Three separate experiments were performed to confirm reproducibility.

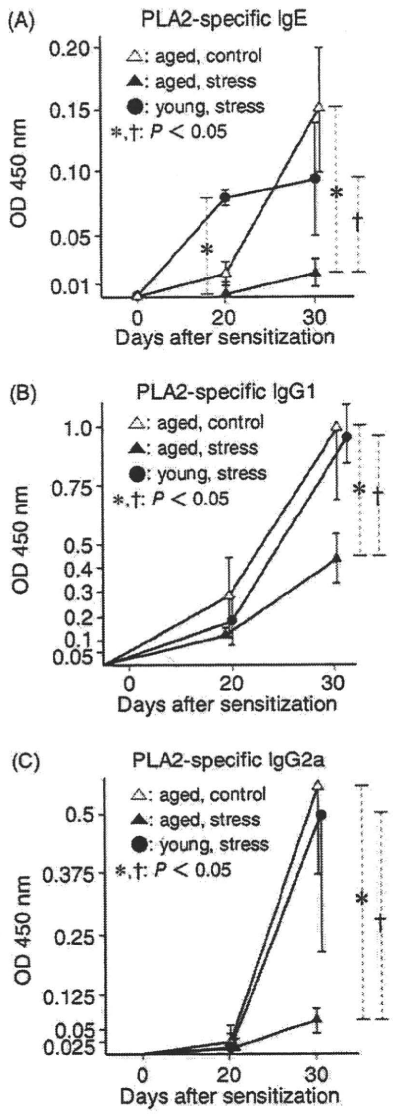

Fig. 2 Effect of restraint stress on PLA2-specific IgE (**A**), IgG1 (**B**) and IgG2a (**C**) production in aged and young mice. Both aged (n = 9, closed triangle) and young (n = 9, closed circle) were placed in a cylindrical chamber for a continuous 8-hour period without food or water. This manipulation was performed once a week, on a total of five occasions. Control aged mice (n = 9, open triangle) were maintained in their cages without food and water at the same time. Blood samples were taken on days 0, 20 and 30 after primary sensitization, and levels of PLA2-specific antibodies were determined by ELISA. Results are expressed as mean ± SEM. Data are representative of 2 separate experiments. *$P < 0.05$ between stressed aged group and control aged group. †$P < 0.05$ between stressed aged group and stressed young group.

PLA2-SPECIFIC IgE, IgG1, AND IgG2a IN SERUM

Serum levels of PLA2-specific IgE, IgG1 and IgG2a were determined using ELISA.[12,14] Titers for specific IgE were estimated as mean optical density (OD) at 450 nm of 1 : 4 diluted sera. Titers for specific IgG1 and IgG2a were estimated as mean OD at 450 nm of 1 : 100 diluted sera.

TOTAL IgE, IgM, AND IgG IN SERUM

Serum levels of total IgE in serum were measured as described previously.[14] The detection limits of this system was 0.3 ng/ml. The levels of total IgM and total IgG were measured using ELISA Quantitation Kit (Bethyl Lavoratories, Inc., Montgomery, TX, USA). The detection limits for IgM and IgG in this system were 0.4 and 0.4 ng/ml, respectively.

CYTOKINE DETERMINATION

Concentration of IL-4, IFN-γ, IL-10 and IL-1β in sera were measured using Opt EIA sets (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA). The detection limits for IL-4, IFN-γ, IL-10 and IL-1β in this system were 10, 60, 15 and 30 pg/ml, respectively.

STATISTICAL ANALYSIS

Data are expressed as means ± standard error of the mean (SEM) for each subject group. Statistical analysis was performed using Student's unpaired t- test to compare titers of PLA2-specific IgE, IgG1 and IgG2a for restrained and control groups. Values of $p < 0.05$ were considered to indicate a statistically significant difference.

RESULTS

EFFECT OF RESTRAINT STRESS ON ANTIGEN-SPECIFIC ANTIBODY PRODUCTION IN AGED MICE

Production of PLA2-specific IgG1 was seen 20 days after the first intranasal sensitization in control aged mice, and production of PLA2-specific IgE and IgG2a, 30 days after the first sensitization. In aged mice under restraint stress, impaired production of these 3 antibodies was observed. On day 30, aged mice under stress produced significantly lower amounts of PLA2-specific IgE, IgG1 and IgG2a as compared with non-stressed aged mice ($P < 0.05$) (Fig. 2A, B, C).

EFFECT OF AGING ON RESTRAINT STRESS-INDUCED INHIBITION OF ANTIGEN-SPECIFIC ANTIBODY PRODUCTION

We then compared PLA2-specific antibody production under restraint stress between young and old mice. Young mice under stress produced PLA2-specific IgE and IgG1 20 days after the first sensitization, and produced PLA2-specific IgG2a 30 days after sensitization. The level of PLA2-specific IgE on day 20 was significantly less in aged mice under stress than young mice, and the difference could still be ob-

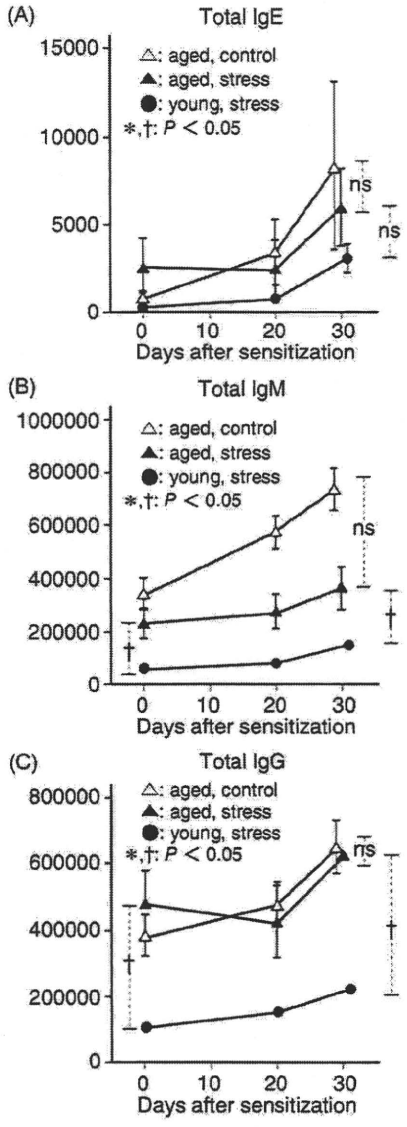

Fig. 3 Effect of restraint stress and/or aging on levels of total IgE (**A**), IgM (**B**), and IgG (**C**) in sera. Both aged (closed triangle) and young (closed circle) mice were placed in cylindrical chambers for a continuous 8-hour period without food or water. This manipulation was performed once a week, on a total of five occasions. Control aged mice (open triangle) were maintained in cages without food and water at the same time. Blood samples were taken on days 0, 20 and 30 after primary sensitisation, and levels of total Ig were determined by ELISA. $*P < 0.05$ between stressed aged group and control aged group. $\dagger P < 0.05$ between stressed aged group and stressed young group.

served on day 30 ($p < 0.05$). In addition, a significant reduction in the production of both PLA2-specific IgG1 and IgG2a was seen on day 30 in aged mice as compared with young mice ($p < 0.05$) (Fig. 2A, B, C).

EFFECTS OF RESTRAINT STRESS ON SERUM LEVELS OF IL-4, IFN-γ, IL-10 AND IL-1β

Serum levels of IL-4, IFN-γ, IL-10 and IL-1β were determined in mice with and without restraint stress. None of these cytokines were detected in sera from non-stressed or stressed aged mice. In addition, these cytokines were not detected even in sera from stressed young mice.

EFFECTS OF AGING AND/OR STRESS ON LEVELS OF TOTAL IgE, IgM, AND IgG IN SERA

Levels of total IgE, total IgM, and total IgG in sera did not differ between stressed aged and non-stressed aged groups. On the other hand, levels of serum total IgM and IgG but not IgE were significantly lower in the stressed young group compared with the stressed aged group throughout the experimental period (Fig. 3).

DISCUSSION

Reductions in T-cell function in aged mice have been shown to reduce IgE antibody production by impairing differentiation of IgE-containing progenitor B cells into IgE antibody-producing plasma cells.[16] These age-associated reductions in immune function, and T-cell function in particular, are thought to affect the function of helper B cells and suppress indirect antibody production response.

In aged mice, various effects of stress in the immune system, and particularly in T cells, have been investigated in previous studies. For example, Kanno *et al.* reported in a study of restraint stress on mice that atrophy of the thymus and decreases in splenic T cells were observed after exposure to stress. However, young mice showed a rapid recovery of the immune function after 1 week, while the aged mice never recovered.[17] However, little is known whether aging can affect stress-induced humoral responses despite the fact that aging and stress share similar effects on immune function.[18]

We previously reported that the humoral immune system in young mice was suppressed by restraint stress in the early stages of antibody production following intranasal sensitization with PLA2.[12] In this study we have further demonstrated that, although repeated intranasal sensitization with PLA2 induced PLA2-specific IgE, IgG1 and IgG2a in aged CBA/J mice, exposure to restraint stress significantly inhibited production of PLA2-specific antibodies. In addition, the present study found that aged mice underwent even more marked suppression of antibody production than young mice under restraint stress. These results suggest for the first time that aging and stress have a synergic effect on the impairment of humoral immunity, and more importantly, that aging exacerbates stress-induced inhibition of humoral re-

sponses. None or only slight differences in antibody production were found between the aged control group and young stressed group. This may be due to an aging effect, and may suggest that the impact of aging on antibody production in our model resembles that of restraint stress seen in young mice.

Restraint stress suppresses both PLA2-specific IgG1 and IgG2a production in aged mice. It is known that IgG1 and IgG2a is Th2 and Th1-type IgG isotype, respectively.[19] Fukui *et al.* reported that restraint stress significantly suppressed both Th1- and Th2-type immune responses in mice.[10] It has also been reported by Dhabhar *et al.* that B cells show a greater stress-induced decrease than T cells.[20] These reports support our findings, suggesting that restraint stress suppresses both Th1- and Th2-type humoral responses in aged mice. Defective induction of functional Th2 cytokine responses has been reported in aged mice[21] in addition to Th1 type immune response being important for the protection against intracellular pathogens such as viruses, mycobacterium and protozoan parasites.[22] Thus susceptibility to impair Th1-type immune responses by restraint stress in elderly patients may increase the risk of suffering from infectious diseases by intracellular pathogens.

The levels of total IgE, total IgM, and total IgG in sera did not differ between stressed aged and non-stressed aged groups. This result suggests that restraint stress selectively affects antigen-specific antibody production in aged mice. Interestingly, levels of serum total IgM and IgG but not IgE were significantly lower in the stressed young group compared with the stressed aged group. This may be due to baseline differences, as serum total IgM and IgG in aged groups were higher than in young groups even before intranasal sensitization. Long-term life in the animal house under a conventional environment may increase serum total IgM and IgG levels.

Although no IL-4, IFN-γ, IL-10 or L-1β was detected in sera from non-stressed aged mice or stressed aged mice, the mechanisms involved in the suppression of antibody production in aged mice under stress have not been clearly elucidated.

Other studies have examined the application of restraint stress, and further studies are needed to clarify the direct or indirect involvement of endocrinological neuronal pathways in the initiation of allergic rhinitis.[23-25] Accumulation of findings from a wide field of research focusing on the immune system and including the nervous endocrine systems is necessary.

In conclusion, we have shown that restraint stress impaired antigen-specific antibody production, especially in aged mice, and aging displays a strong impact on stress-induced inhibition of humoral immune responses. These observations may provide a basis for the management of care for elderly patients with physical restraints. In modern life, both the young and elderly are exposed to various forms of stress.[26] Our study suggests stress as one of the mechanisms for the epidemiological finding that serum IgE levels and antigen-specific IgE production decline with age in humans.[6,7]

ACKNOWLEDGEMENTS

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CRTH2 Plays an Essential Role in the Pathophysiology of Cry j 1-Induced Pollinosis in Mice[1]

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PGD_2 is the major prostanoid produced during the acute phase of allergic reactions. Two PGD_2 receptors have been isolated, DP and CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 cells), but whether they participate in the pathophysiology of allergic diseases remains unclear. We investigated the role of CRTH2 in the initiation of allergic rhinitis in mice. First, we developed a novel murine model of pollinosis, a type of seasonal allergic rhinitis. Additionally, pathophysiological differences in the pollinosis were compared between wild-type and *CRTH2* gene-deficient mice. An effect of treatment with ramatroban, a CRTH2/T-prostanoid receptor dual antagonist, was also determined. Repeated intranasal sensitization with Cry j 1, the major allergen of *Cryptomeria japonica* pollen, in the absence of adjuvants significantly exacerbated nasal hyperresponsive symptoms, Cry j 1-specific IgE and IgG1 production, nasal eosinophilia, and Cry j 1-induced in vitro production of IL-4 and IL-5 by submandibular lymph node cells. Additionally, CRTH2 mRNA in nasal mucosa was significantly elevated in Cry j 1-sensitized mice. Following repeated intranasal sensitization with Cry j 1, *CRTH2* gene-deficient mice had significantly weaker Cry j 1-specific IgE/IgG1 production, nasal eosinophilia, and IL-4 production by submandibular lymph node cells than did wild-type mice. Similar results were found in mice treated with ramatroban. These results suggest that the PGD_2-CRTH2 interaction is elevated following sensitization and plays a proinflammatory role in the pathophysiology of allergic rhinitis, especially pollinosis in mice. *The Journal of Immunology,* 2008, 180: 5680–5688.

Pollinosis, a type of seasonal allergic rhinitis, is the most common allergic respiratory disease and is a global health problem that is increasing in prevalence (1–3). For example, as much as 10–20% of the Japanese population suffers from Japanese cedar pollinosis (JCP).[3]

Intensive and extensive studies on pollinosis have greatly improved the understanding of its etiology and pathology (4). Mouse models of allergic rhinitis have contributed to these advances. However, these mouse models usually use adjuvants and/or strong Ags to efficiently sensitize animals (5–9). To further examine the pathophysiological mechanism underlying pollinosis, a murine model that naturally mimics human pollinosis by intranasal administration of pollen extracts in the absence of adjuvants is needed.

Prostanoids are thought to participate in allergic inflammation (10). PGD_2 is one of the most important of these and it plays roles in allergic respiratory diseases including allergic rhinitis (10–15). For example, nebulized PGD_2 enhances Th2-type inflammatory responses and eosinophilia, leading to the development of airway hyperresponsiveness (14). PGD_2 acts via the D-prostanoid receptor (DP) and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) (16). The expression patterns and signaling pathways utilized by DP and CRTH2 are different, suggesting that they have distinct roles in allergic responses (16, 17). It appears that signals via DP promote eosinophil survival, whereas signals via CRTH2 mediate shape changes, chemotaxis, and degranulation by eosinophils (16, 18, 19).

The role of CRTH2 in allergic airway inflammation in vivo remains controversial (17). CRTH2 has been found to participate in the recruitment of eosinophils from the bone marrow into the bloodstream (19, 20), in eosinophilic airway inflammation (11), and in airway eosinophilia and hyperresponsiveness (21), suggesting that it plays a proinflammatory role in vivo. On the other hand, mice deficient for CRTH2 ($CRTH2^{-/-}$) show eosinophil recruitment and IL-5 production by splenocytes in an asthma model, suggesting that CRTH2 mediates antiinflammatory signals (22). In human nasal mucosa, CRTH2 is expressed in eosinophils and a subset of T cells (23). We have recently reported that there is a close correlation between the number of eosinophils infiltrating into nasal mucosa and the amount of CRTH2, but not DP, in nasal mucosa (12). Also, $CRTH2^{-/-}$ mice have been found to show reduced eosinophil infiltration into skin in a model of chronic allergic skin inflammation (24).

In this study, we established a novel murine model of pollinosis and used it to determine the pathophysiological role of CRTH2 in

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[3] Abbreviations used in this paper: JCP, Japanese cedar pollinosis; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; DP, D-protanoid receptor; TP, T-prostanoid receptor; WT, wild type.

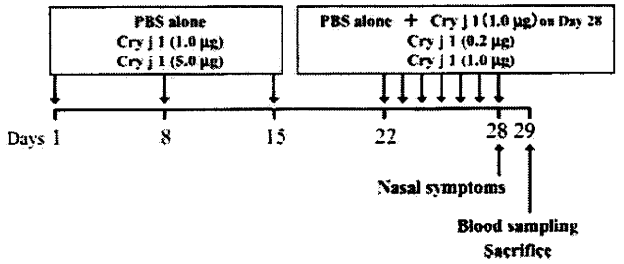

FIGURE 1. Experimental design used to investigate the effect of nasal exposure to Cry j 1 in mice. BALB/c mice (6–9 per group) were sensitized by intranasal administration of either 1.0 or 5.0 μg of Cry j 1 in 10 μl PBS in the absence of adjuvants once a week for 3 wk (on days 1, 8, and 15). One week after the third sensitization, mice were challenged by intranasal administration of one fifth Cry j 1, respectively every day for 7 consecutive days (on days 22 to 27). As a control, mice were treated with PBS, except for the final challenge, where mice were treated with 1.0 μg of Cry j 1. Immediately after the final nasal challenge, nasal symptoms were observed for 10 min, and 16 h after the final nasal challenge, peripheral blood was collected, and the specific Ab content in the serum was measured. After the blood sampling, mice were sacrificed, and the nose and submandibular lymph nodes were obtained for further analysis.

the disease. In this model, intranasal sensitization with Cry j 1, the major allergen of *Cryptomeria japonica* pollen, in the absence of adjuvant induced allergic rhinitis closely resembling human pollinosis. We found that a lack of CRTH2 in the mutant mice greatly reduces allergic pathophysiologies in this model.

Materials and Methods

Animals and Ags

BALB/c mice were purchased from Charles River Laboratories Japan and CLEA Japan. Homozygous CRTH2-deficient and wild-type BALB/c mice were obtained as described previously (24). Female mice (7–11 wk old) were used in all the experiments. The mice were maintained in specific pathogen-free conditions at Okayama University and Tokyo Medical and Dental University in accordance with the guidelines set forth by the university committees. All experimental protocols and procedures in the present study were approved by the University Animal Care and Use Committees. Cry j 1 was purified from crude extracts of *C. japonica* pollen as described previously (25). Endotoxin contamination was considered to be negligible due to a negative result in the Endospec ES test (Seikagaku). Ramatroban was obtained from Bayer Yakuhin. Protein concentrations were determined using a bicinchoninic acid assay (Pierce) according to the manufacturer's instructions.

Sensitization of mice

Mice (6–9 animals per group) were sensitized by intranasal application of serial doses of Cry j 1 in 10 μl PBS in the absence of adjuvants using a microsyringe (Hamilton Medical). The low-dose sensitization consisted of a series of administrations of 1.0 μg of Ag once a week for 3 wk (on days 1, 8, and 15), followed by administration of 0.2 μg Ag every day for 7 consecutive days (on days 22 to 28). For high-dose sensitization, 5.0 and 1.0 μg of Cry j 1 were administered once a week for 3 wk (on days 1, 8, and 15) and every day for 7 consecutive days (on days 22 to 28), respectively. As a control, mice were treated with PBS instead of the Ag at all points except for the final challenge, where the mice were administered 1.0 μg of Cry j 1 (Fig. 1). Immediately after the final nasal challenge, the frequencies of sneezing and nasal rubbing were counted in a blinded manner for 10 min. Peripheral blood was collected from the tail vein 16 h after the final nasal challenge, and then sera were prepared by centrifugation at 200 × *g*, and the levels of Cry j 1-specific Ab in the serum were determined by ELISA. The mice were then sacrificed, and the nose and submandibular lymph nodes were isolated for further immunological and histological analyses.

To determine whether the effect of CRTH2 deficiency is at the level of sensitization or amplification of allergic cascade, outcomes of pollinosis were compared with CRTH2$^{-/-}$ mice sensitized and subsequently challenged with Cry j 1 and nonsensitized CRTH2$^{-/-}$ mice with a single challenge with Cry j 1.

Ramatroban treatment

Ramatroban was suspended in 5% methyl cellulose and administered orally at a dose of 30 mg/kg body weight once a day from 1 day before the first sensitization to the final challenge (day 0 to day 28). Control mice were given 5% methyl cellulose alone.

Ab determination

The levels of Cry j 1-specific IgE, IgG1, and IgG2a were determined by ELISA as previously described (26). The levels of Cry j 1-specific IgE were measured using biotinylated Cry j 1 (Hayashibara Biochemical Laboratories) as a detecting reagent. The titers of Ag-specific Abs were estimated according to the mean OD at 450 nm of serum dilutions of 1/20 for IgE and 1/100 for IgG1 and IgG2a.

In vitro culture of submandibular lymph node cells and measurement of cytokine production

Submandibular lymph nodules from mice were dispersed and filtered through a 70-μm cell strainer (BD Biosciences) to yield a single-cell suspension. Lymph node cells were suspended in RPMI 1640 supplemented with 10% heat-inactivated FCS (Invitrogen), 100 μg/ml streptomycin, 100 U/ml penicillin, and 20 mM L-glutamine (Sigma-Aldrich). Cells (4×10^5 cells/200 μl) were cultured in the presence or absence of 10 μg/ml Cry j 1 in 96-well flat-bottom plates (BD Biosciences) at 37°C in humidified atmosphere of 5% CO_2 and 95% air. After 72 h of culture, supernatants were harvested. The levels of IL-4, IL-5, and IFN-γ in the culture supernatant were measured using OptEIA sets (BD Biosciences). The levels of IL-13 were measured using DuoSet ELISA development kit (R&D Systems). The detection limits for IL-4, IL-5, IL-13, and IFN-γ in this system were 10, 30, 40, and 60 pg/ml, respectively.

Histological examination

Histological examination was performed as previously described (26). Coronal nasal sections were stained with H&E and Luna solution to detect mononuclear cells and eosinophils, respectively. A blind test was conducted to determine the numbers of infiltrating cells in the posterior part of nasal septum using a high-power (10 × 40) microscopic field.

To determine the infiltration of T cells into nasal mucosa, immunohistochemistry for CD3 was examined. Paraffin-embedded nasal tissues were sectioned into 5-μm slices, deparaffinized, rehydrated and retrieved with microwave. Endogenous peroxidase activity was quenched with 3% H_2O_2, and nonspecific protein binding was blocked with normal rabbit serum (DAKO Japan) for 60 min. After this, the tissue sections were incubated with goat anti-mouse CD3-ε polyclonal Ab (sc-1127; Santa Cruz Biotechnology) or control goat IgG Ab (M-20; Santa Cruz Biotechnology) overnight at 4°C. To detect the reaction, N-Histofine Simple Stain MAX PO (G) (Nichirei Biosciences) and diaminobenzidine substrate (DAKO Japan) was used according to the manufacturers' instructions.

Real-time quantitative PCR in nasal mucosa

Mucosal tissues were removed from nasal septum 16 h following the final nasal challenge, immediately soaked in buffer containing guanidine isothiocyanate from the RNeasy Mini Kit (Qiagen), and stored at −80°C until use. Extraction of total cellular RNA, reverse transcription to generate cDNA, and real-time quantitative PCR were performed using a Chromo4 Real-Time PCR detector (Bio-Rad Laboratories, Hercules) and QuantiTect SYBR Green PCR reagents (Qiagen) as described previously (12). The primer sequences for CRTH2 and GAPDH are shown in Table I. Standard curves for both CRTH2 and GAPDH were generated using a PCR fragment of CRTH2 and plasmid DNA of GAPDH as a standard, respectively. Then absolute copy number of CRTH2 and GAPDH for each sample was calculated, and samples were reported with a CRTH2 copy number relative to GAPDH.

Relative amounts of IL-4, IL-5, IL-13, IFN-γ, IL-1β, IL-6, TNF-α, RANTES, and eotaxin mRNA in nasal mucosa were also measured. The primers used are listed in Table I.

Statistical analysis

Statistical significance was determined by nonparametrical Mann-Whitney *U* tests. p values of <0.05 were considered to indicate statistical significance. Values are shown as means ± SEM.

Table I. *Primary sequences used for real-time PCR amplifications*

	Forward Primer	Reverse Primer	Amplification Size (bp)	Genbank Accession No.
IL-4	CCTCACAGCAACGAAGAACA	CTGCAGCTCCATGAGAACAC	133	NM_021283
IL-5	TCAGCTGTGTCTGGGCCACT	TTATGAGTAGGGACAGGAAGCCTCA	133	NM_010558
IL-13	TGCTTGCCTTGGTGGTCTC	CAGGTCCACACTCCATACC	151	NM_008355
IFN-γ	GCGTCATTGAATCACACCTG	ACCTGTGGGTTGTTGACCTC	103	NM_008337
IL-1β	TCCAGGATGAGGACATGAGCAC	GAACGTCACACCAGCAGGTTA	105	NM_008361
IL-6	CCACTTCACAAGTCGGAGGCTTA	GCAAGTGCATCATCGTTGTTCATAC	112	NM_031168
TNF-α	ATGAGCACAGAAAGCATGATC	TCCACTTGGTGGTTTGCTACG	305	NM_013693
RANTES	AGATCTCTGCAGCTGCCCTCA	GGAGCACTTGCTGCTGGTGTAG	170	NM_013653
Eotaxin	CAGATGCACCCTGAAAGCCATA	TGCTTTGTGGCATCCTGGAC	96	NM_011330
CRTH2	TCTCAACCAATCAGCACACC	CCTCCAAGAGTGGACAGAGC	173	NM_009962
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	452	NM_008084

Results

Induction of nasal symptoms in Cry j 1-sensitized mice

We first attempted to generate a mouse model mimicking human allergic rhinitis, especially pollinosis, which causes symptoms of nasal symptoms, including sneezing and nasal rubbing, by intranasal administration of Cry j 1. We found a significant and dose-dependent increase in the frequency of sneezing in BALB/c mice sensitized with Cry j 1. Mice that were treated with PBS alone sneezed 1.8 ± 0.3 (mean ± SEM) times in the 10 min following the final Ag administration, whereas they sneezed 5.8 ± 1.2 times and 15.7 ± 2.7 times when treated with low and high doses of Ag, respectively (Fig. 2*A*). Similarly, immediately after the final Ag challenge, nasal rubbing was observed more frequently in mice sensitized with a high dose of Cry j 1 than in control mice (37.3 ± 5.8 vs 11.2 ± 2.7 times in 10 min). At a low dose of Cry j 1, there was no significant increase in the frequency of nasal rubbing (Fig. 2*B*).

Development of Th2-type immune responses in Cry j 1-sensitized mice

To further characterize the pathogenesis of immune responses caused by Cry j 1, we monitored several parameters associated with pollinosis. Nasal challenge with a low or high dose of Cry j 1 caused a considerable increase in the concentration of Cry j 1-specific IgE in sera when measured 1 day after the final challenge (Fig. 2*C*). There was also a significant elevation in the concentration of Cry j 1-specific IgG1 (Fig. 2*D*). The concentration of Cry j 1-specific IgE and IgG1 was not appreciably different at the low and high doses of Cry j 1. Cry j 1, however, had little effect on the level of Cry j 1-specific IgG2a (Fig. 2*E*).

Eosinophil infiltration into nasal mucosa, another characteristic of pollinosis, is rarely seen in the nasal mucosa in control mice (Fig. 3*A*). On the contrary, there was a marked accumulation of eosinophils not only in the lamina propria but also in the epithelial layer in mice 1 day after the final challenge (Fig. 3, *B* and *C*). Eosinophil numbers per field following intranasal Cry j 1 sensitization/challenge at both low and high doses were significantly higher than in control mice (Fig. 3*D*). The nasal mucosa of Cry j 1-sensitized mice also showed severe infiltration by mononuclear cells. The nasal septum of mice treated with low and high doses of Cry j 1 contained more mononuclear cells per field (59.8 ± 9.0 ($p = 0.055$) and 80.2 ± 9.1 ($p = 0.016$), respectively) than did control mice (39.8 ± 4.7).

We next examined the in vitro production of cytokines in culture by cells isolated from submandibular lymph nodes from mice treated in vivo with or without Cry j 1. The amounts of IL-4 and

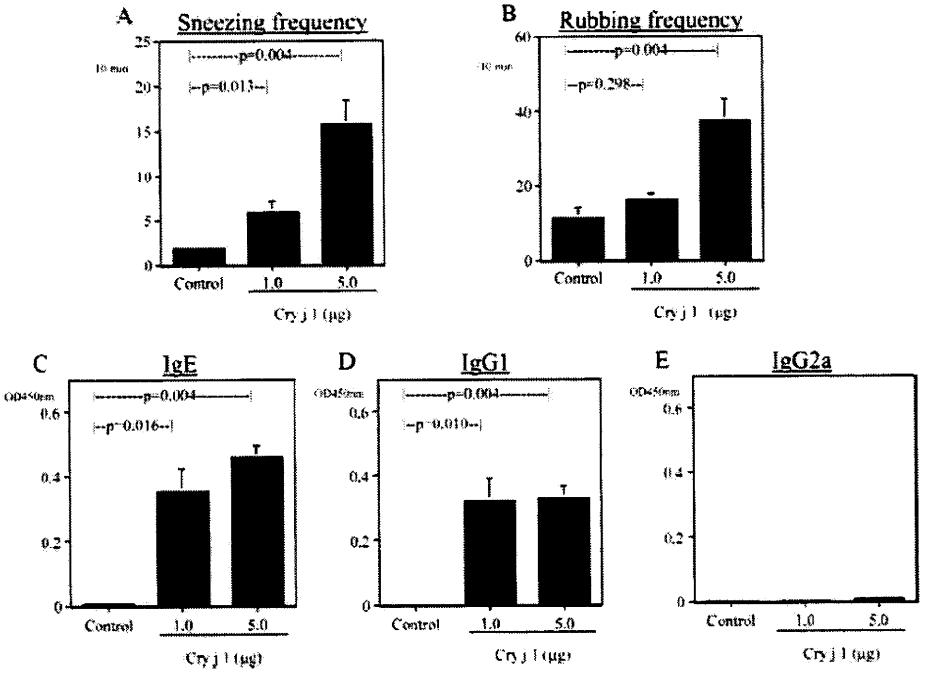

FIGURE 2. Nasal hyperresponsive symptoms and Ab production in mice following intranasal sensitization and challenge with Cry j 1. Mice were sensitized and challenged by intranasal administration of Cry j 1. Nasal allergic symptoms, including the frequency of sneezing (*A*) and rubbing (*B*), were determined for the 10 min immediately following the final nasal challenge (day 28). Mean frequencies ± SEM are shown. Serum samples were obtained 16 h after the final intranasal challenge. Cry j 1-specific IgE (*C*), IgG1 (*D*), and IgG2a (*E*) levels were determined by ELISA. Mean OD values ± SEM are shown. Results are representative of two independent experiments.

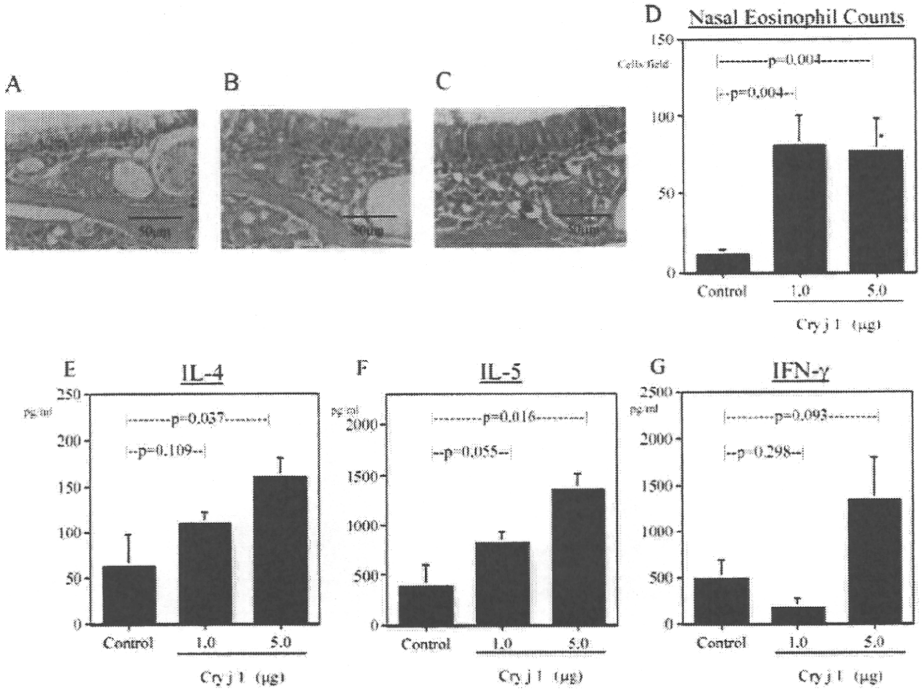

FIGURE 3. Nasal eosinophilia and cytokine production by submandibular lymph node cells following intranasal sensitization and challenge with Cry j 1. Mice were sensitized and challenged by intranasal administration of PBS (*A*), low-dose Cry j 1 (*B*), or high-dose Cry j 1 (*C*) according to the schedule shown in Fig. 1. Sixteen hours after the final challenge, nasal sections were collected, fixed, and decalcified, and eosinophils in the nasal mucosa were detected by Luna stain. *D*, The numbers of eosinophils in the posterior portion of the nasal septum were determined per high-power (10 × 40) microscopic field. Mean numbers of infiltrating cells per field ± SEM are shown. Sixteen hours after the final challenge, submandibular lymph node cells were isolated and cultured in the absence or presence of Cry j 1 for 72 h. IL-4 (*E*), IL-5 (*F*), and IFN-γ (*G*) were measured by ELISA. Mean concentrations ± SEM are shown. Results are representative of two independent experiments.

IL-5 produced by the cells were in proportion to the doses used for in vivo sensitization (Fig. 3, *E* and *F*). IFN-γ production was slightly enhanced in lymph node cells from mice treated with a high dose of Cry j 1 compared with control mice, but the increase was not statistically significant (Fig. 3*G*).

CRTH2 mRNA expression in nasal mucosa of Cry j 1-sensitized mice

We next measured the expression of CRTH2 at sites of nasal inflammation. Control mice treated with PBS expressed a low level of CRTH2 mRNA in the mucosal tissue of the nasal septum. In mice treated with Cry j 1, the level of CRTH2 mRNA was significantly increased (Fig. 4). Thus, we further investigated whether CRTH2 is positively or negatively involved in the pathophysiology of pollinosis using CRTH2$^{-/-}$ mice.

Impaired pathophysiology of pollinosis in Cry j 1-sensitized CRTH2$^{-/-}$ mice

A high dose of Cry j 1 was administered to both wild-type (WT) and CRTH2$^{-/-}$ mice, and the nasal hyperresponsive symptoms were examined immediately after the final nasal challenge. Notably, the number of sneezes in 10 min by the Cry j 1-sensitized mutant mice was significantly lower than by the WT mice (Fig. 5*A*). Nasal rubbing was also significantly lower in the CRTH2$^{-/-}$ mice than in the WT mice (Fig. 5*B*).

The level of Cry j 1-specific IgE in serum samples collected on the day following the final Ag challenge was significantly lower for mutant mice than for WT mice (Fig. 5*C*). Production of Cry j 1-specific IgG1 was similarly reduced in CRTH2$^{-/-}$ mice compared with WT mice (Fig. 5*D*). In contrast, serum levels of Cry j 1-specific IgG2a were the same in the two mouse strains (Fig. 5*E*).

The number of eosinophils infiltrating into the nasal septum following administration of Cry j 1 was also significantly lower in the CRTH2$^{-/-}$ mice than in the WT mice (Fig. 6*A–C*). Although the number of mononuclear cells infiltrating the nasal septum was not significantly different in the mutant and WT mice (Fig. 6*D*), the number of infiltrating CD3$^+$ cells was significantly reduced in CRTH2$^{-/-}$ mice as compared with WT mice (Fig. 6*E*). These

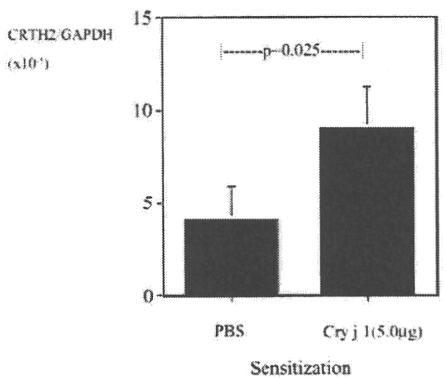

FIGURE 4. Relative amounts of CRTH2 mRNA in nasal mucosa. Mice (6 per group) were sensitized by intranasal administration of 5.0 μg of Cry j 1 once a week for 3 wk. One week after the third sensitization, mice were challenged by intranasal administration of 1.0 μg of Cry j 1 each day for 7 consecutive days. Control animals were treated with PBS at all steps except for the final challenge, where they were treated with 1.0 μg of Cry j 1. Sixteen hours after the final challenge with Cry j 1, mucosal tissues were removed from the nasal septum. The CRTH2 mRNA levels were estimated using real-time quantitative PCR. Results are the mean amounts of mRNA ± SEM.

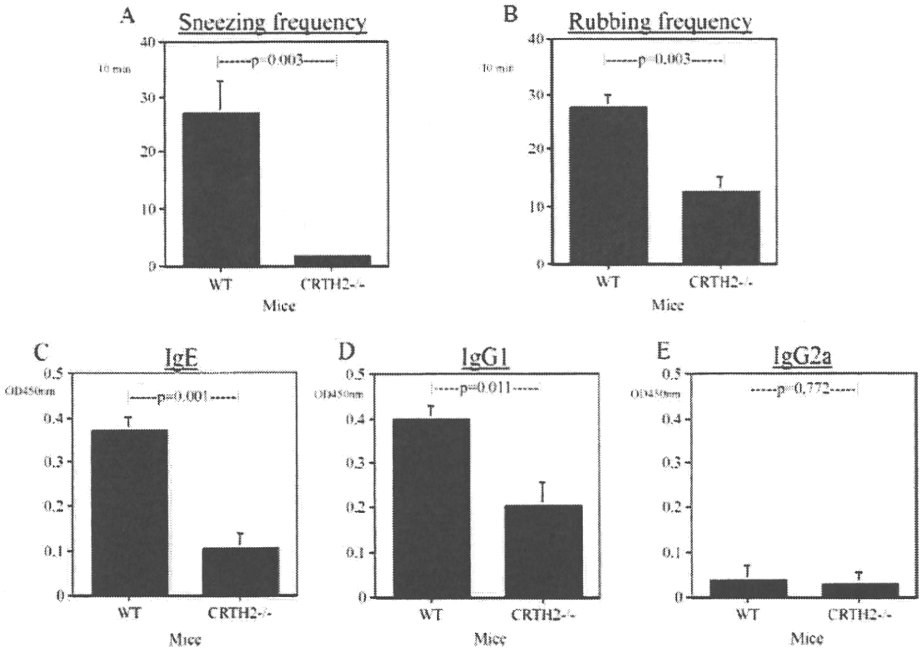

FIGURE 5. Nasal symptoms and Ab production in WT and CRTH2$^{-/-}$ mice following the final nasal challenge with Cry j 1. Mice were sensitized and challenged by intranasal administration of Cry j 1. Sneezing (*A*) and rubbing (*B*) frequency were measured for 10 min following the final nasal challenge (day 28). Mean frequencies ± SEM are shown. Sixteen hours after the final nasal challenge, blood was sampled from mice, and levels of serum Cry j 1-specific IgE (*C*), IgG1 (*D*), and IgG2a (*E*) were determined by ELISA. Mean ODs ± SEM are shown. Results are representative of two independent experiments.

results suggest that CRTH2 deficiency affects infiltration of not only eosinophils but also T cells.

To clarify the link between CRTH2 deficiency and the relief of allergic symptoms, we further investigated cytokine production in vitro by cells from submandibular lymph nodes obtained the day after the final Ag challenge. The amount of IL-4 was 5-fold lower in CRTH2$^{-/-}$ mice than in WT mice (Fig. 6*F*). Additionally, there was a slight reduction in the amount of IL-5 (Fig. 6*G*) and a slight increase in the amount of INF-γ (Fig. 6*I*) in the cells from CRTH2$^{-/-}$ mice, but the differences were not significant. On the contrary, the levels of IL-13 were significantly higher in CRTH2$^{-/-}$ mice as compared with WT mice (Fig. 6*H*).

Additionally, mRNA levels of Th2 cytokines (IL-4, IL-5, and IL-13), Th1 cytokine (IFN-γ), proinflammatory cytokines (IL-1β, IL-6 and TNF-α), and eosinophil-chemotactic chemokines (RANTES and eotaxin) in nasal mucosa were determined. The

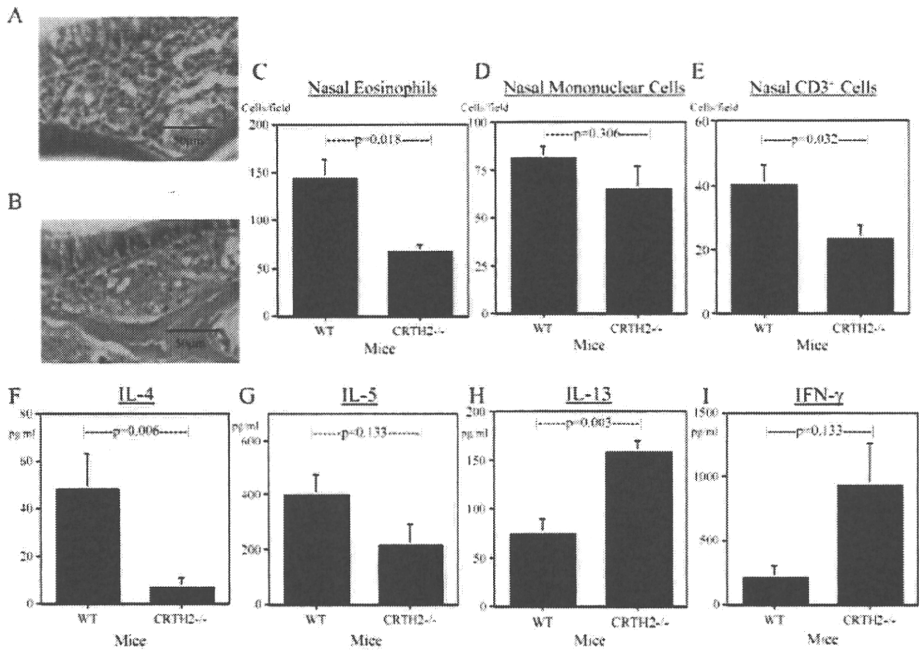

FIGURE 6. Histological changes and cytokine production by submandibular lymphocytes following nasal challenge with Cry j 1 in WT and CRTH2$^{-/-}$ mice. WT (*A*) and CRTH2$^{-/-}$ (*B*) mice were sensitized and challenged by intranasal administration of Cry j 1. Sixteen hours following the final nasal challenge with Cry j 1, nasal sections were collected, fixed, and decalcified, and eosinophils in nasal mucosa were detected by Luna stain. *C*, The number of eosinophils in the posterior portion of the nasal septum was determined per high-power (10 × 40) microscopic field. Mean numbers of infiltrating eosinophils per field ± SEM are shown. Numbers of mononuclear cells (*D*) and CD3$^+$ cell (*E*) in the nasal septum were also determined. Sixteen hours after the final challenge with Cry j 1, submandibular lymph node cells were isolated and cultured with Cry j 1 for 72 h. IL-4 (*F*), IL-5 (*G*), IL-13 (*H*), and IFN-γ (*I*) were measured by ELISA. Mean concentrations ± SEM are shown. Results are representative of two independent experiments.

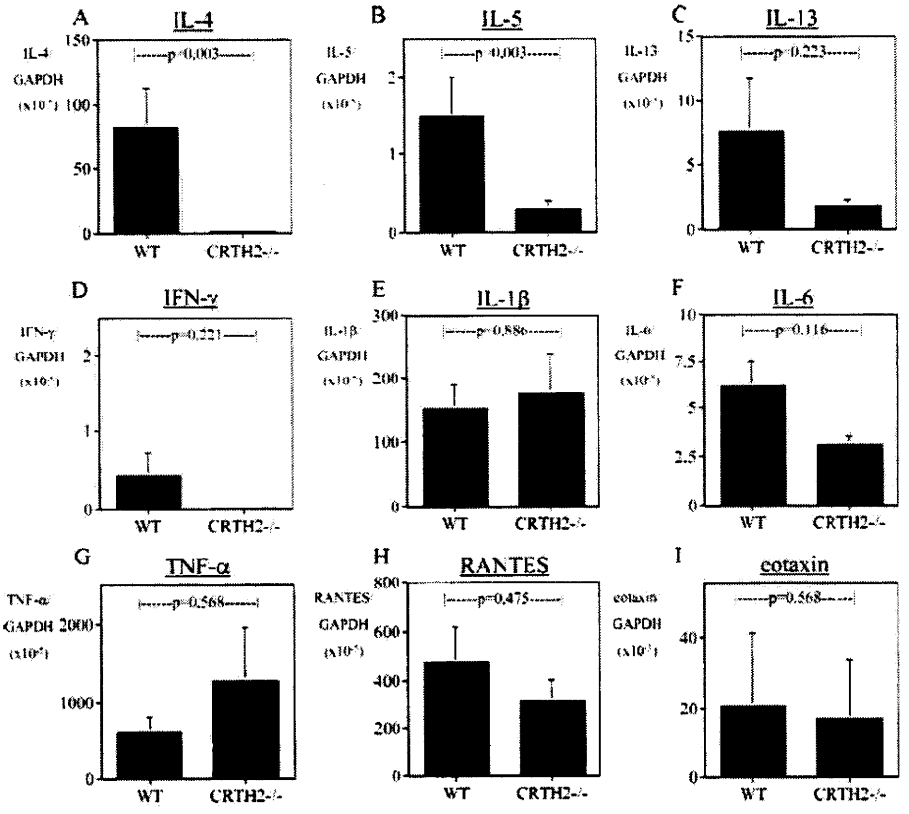

FIGURE 7. Relative amounts of cytokines/chemokines mRNA in nasal mucosa following nasal challenge with Cry j 1 in WT and CRTH2$^{-/-}$ mice. Sixteen hours after the final nasal challenge with Cry j 1, mucosal tissues were removed from nasal septum. Relative amounts of IL-4 (*A*), IL-5 (*B*), IL-13 (*C*), IFN-γ (*D*), IL-1β (*E*), IL-6 (*F*), TNF-α (*G*), RANTES (*H*), and eotaxin (*I*) mRNA were compared between WT and CRTH2$^{-/-}$ mice. Results are the mean amounts of mRNA ± SEM.

levels of IL-4 and IL-5 mRNA were significantly lower in CRTH2$^{-/-}$ mice as compared with WT mice, whereas the levels of other cytokines/chemokines were similar between CRTH$^{-/-}$ and WT mice (Fig. 7). These results suggest that reduced nasal eosinophilia in CRTH2 deficiency is associated with reduced levels of IL-5 but not RANTES or eotaxin in this model. Additionally,

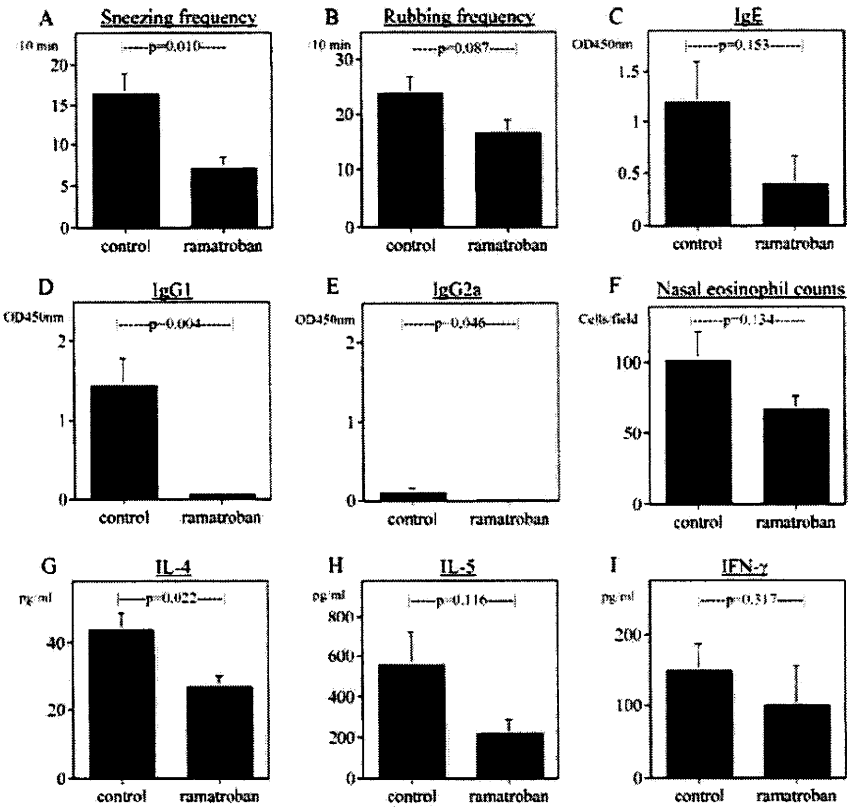

FIGURE 8. Effects of ramatroban on murine JCP. Ramatroban (30 mg/kg body weight), suspended in 5% methyl cellulose, was given orally once a day from 1 day before the first sensitization to the final challenge (day 0 to day 28). Control mice were given 5% methyl cellulose alone. After the final intranasal challenge, the frequencies of sneezing (*A*) and rubbing (*B*) were counted, and serum levels of Cry j 1-specific IgE (*C*), IgG1 (*D*), IgG2a (*E*), and nasal eosinophil count (*F*), as well as Cry j 1-induced IL-4 (*G*), IL-5 (*H*) and IFN-γ (*I*) were determined as described in *Materials and Methods*. Results are expressed as means ± SEM.

it is suggested that CRTH2-mediated pathway may induce pathology without regulating local production of these proinflammatory cytokines.

Outcomes of pollinosis were compared between sensitized/challenged CRTH2$^{-/-}$ mice and nonsensitized/single-challenged CRTH2$^{-/-}$ mice. The levels of Cry j 1-specific IgE (0.159 ± 0.044 vs 0 ± 0 OD at 450 nm: $p = 0.003$), Cry j 1-specific IgG1 (0.638 ± 0.163 vs 0 ± 0 OD at 450 nm: $p = 0.004$), nasal eosinophila (66.4 ± 8.2 vs 6.6 ± 1.1 cells/field: $p = 0.005$), and IL-4 production by submandibular lymph node cell (72.8 ± 31.1 vs 6.7 ± 3.8 pg/ml: $p = 0.004$) were significantly higher in sensitized and subsequently challenged CRTH2$^{-/-}$ mice as compared with nonsensitized and single-challenged CRTH2$^{-/-}$ mice. However, the frequencies of sneezing (1.7 ± 0.5 vs 0.6 ± 0.2 times in 10 min: $p = 0.088$) and rubbing (12.3 ± 2.6 vs 10.2 ± 3.1 times in 10 min: $p = 0.516$) were similar between two groups, suggesting that CRTH2 is particularly essential for the development of nasal symptoms.

Effect of ramatroban on Cry j 1-induced pollinosis

As seen in CRTH2-deficient mice, treatment with ramatroban significantly reduced several indicators of pollinosis including sneezing, Cry j 1-specific IgG1 production, and Cry j 1-induced IL-4 production by submandibular lymph node cells as compared with the control treatment (Fig. 8, *A*, *D*, and *G*). Although the differences did not reach to the statistical level, other parameters such as nasal rubbing, Cry j 1-specific IgE production, nasal eosinophilia, and Cry j 1-induced IL-5 production were also reduced by the treatment with ramatroban (Fig. 8, *B*, *C*, *F*, and *H*).

Discussion

In the present study, we analyzed the pathophysiological effects of nasal exposure to Cry j 1 in BALB/c mice. Mice sensitized with Cry j 1 without adjuvants showed not only allergic symptoms such as sneezing and rubbing but also produced Cry j 1-specific IgE and IgG1 and displayed nasal eosinophilia. Additionally, submandibular lymph node cells isolated from these mice produced IL-4 and IL-5 in recall response to Cry j 1. These results suggest that intranasal sensitization with Cry j 1 induces pollinosis in BALB/c mice.

To investigate the initiation of allergic rhinitis in vivo, administration of Ags via the natural route (i.e., through the nostril) is desirable. In fact, it is known that administration of Ags through different routes results in different degrees of IgE production (27, 28). Also, murine models of allergic rhinitis have been generated by intranasal or aerosol-mediated sensitization (8, 29), but these models generally employ adjuvants such as cholera toxin, which have immunoregulatory effects that may distort the physical sensitization (30, 31). Therefore, we and others have established murine models of allergic rhinitis by intranasal sensitization with Ags including *Schistosoma mansoni* egg Ag, phospholipase A_2 from honeybee venom, extracts of *Aspergillus fumigatus*, OVA, and trimellitic anhydride in the absence of adjuvants (5–7, 9, 32). We think that our current model is the first in which murine pollinosis was induced by intranasal sensitization with pollen allergen in the absence of an adjuvant. This model may be useful not only for understanding the pathophysiology of pollinosis but also for developing and/or testing new therapies for allergic rhinitis, especially JCP.

BALB/c mice sensitized with Cry j 1 showed an increase in the expression of CRTH2 mRNA in the nasal septum compared with control mice. This agrees with our recent report demonstrating that the amount of CRTH2 mRNA in nasal mucosa is significantly higher in patients with allergic rhinitis than in control subjects not showing hypertrophy of inferior turbinates (12). These results suggest that the expression of CRTH2 may play a role in the pathogenesis of allergic rhinitis both in humans and in mice. In fact, it is known that the expression of CRTH2 in eosinophils and CD4$^+$ T cells is elevated in atopic patients (33–35). CRTH2 is expressed by eosinophils and a subset of CD3$^+$ T cells in nasal mucosa, especially in patients with allergic rhinitis (23). Because a mAb against murine CRTH2 that can be used for immunohistochemistry is not currently available, we could not investigate the phenotype of cells expressing CRTH2 in mice.

The pathophysiology of allergic rhinitis was clearly impaired in CRTH2$^{-/-}$ mice. Following repeated intranasal sensitization and nasal challenge with Cry j 1, CRTH2$^{-/-}$ mice displayed reduced nasal symptoms, production of Cry j 1-specific IgE and IgG1, and nasal eosinophilia compared with WT mice. Additionally, submandibular lymph node cells from Cry j 1-sensitized CRTH2$^{-/-}$ mice produced significantly less IL-4 and IL-5 in response to Cry j 1 than those from WT mice. We think that the present results are the first demonstration of the in vivo role of CRTH2 in the initiation of Th2 responses in the upper airway.

We also found that Cry j 1-specific IgE and IgG1 but not IgG2a production was impaired in CRTH2$^{-/-}$ mice. Ag-specific IgE/IgG1 and IgG2a production is known to be positively regulated by Th2 and Th1 responses, respectively, in mice (36). Thus, our results indicate that signals mediated by CRTH2 selectively enhance Th2-type Ab production. The decreased production of IL-4 by submandibular lymph node cells from CRTH2$^{-/-}$ mice in response to Cry j 1 restimulation supports this result because IL-4 plays a critical role in IgE synthesis in vivo (37). Although whether CRTH2 activation directly leads to IL-4 production in mice remains unclear, recent investigations have demonstrated that PGD_2 causes the preferential induction of IL-4 production by Th2 cells in humans by binding to CRTH2 (38, 39). Additionally, our recent report showing that CRTH2 signals up-regulate CD40L in resting human Th2 cells supports our conclusions because the engagement of CD40 by CD40L is also essential for IgE isotype switching (39, 40).

After intranasal sensitization with Cry j 1, CRTH2$^{-/-}$ mice developed a weaker eosinophilia than did WT BALB/c mice. This suggests that CRTH2 mediates local eosinophil recruitment in this model, which agrees with reports showing that CRTH2 activation leads to changes in eosinophil shape, chemotaxis, and degranulation in vitro (16, 18, 41). Additionally, recent investigations have revealed that CRTH2 plays a proinflammatory role in eosinophil chemotaxis into inflamed tissue in vivo (11, 12, 21, 24, 42). On the other hand, submandibular lymph node cells from WT and CRTH2$^{-/-}$ mice produced similar amount of IL-5 after intranasal sensitization with Cry j 1. It is well known that IL-5 plays a critical role in eosinophilic inflammation, especially in mice (43). Although little is known about whether CRTH2 activation enhances IL-5 production in mice, CRTH2 activation on Th2 cells is known to induce IL-5 production in humans (38, 39). One explanation of why nasal eosinophilia was reduced in CRTH2$^{-/-}$ mice irrespective of IL-5 production is that cognate interaction between PGD_2 and CRTH2 on eosinophils may have an additive effect on local eosinophil recruitment, primarily due to the action of IL-5. In fact, in a mouse model of asthma, nebulized DK-PGD_2, a CRTH2 agonist, exacerbates eosinophilic lung inflammation without changes in IL-5 content in lung (21).

CRTH2$^{-/-}$ mice displayed a significantly lower frequency of both sneezing and nasal rubbing after the nasal challenge compared with the WT mice. Several molecules, including IL-5, CD80/CD86, H1, and CD39, have been shown to contribute to these symptoms via different mechanisms (38, 44–46). The present result suggests that activation of CRTH2 is also involved

in the symptoms of nasal hyperreactivity. In humans, nasal challenge with PGD_2 induces a sustained nasal obstruction but not sneezing or rhinorrhea (47). Whether murine mast cells express CRTH2 is not well known, and further investigations are needed to determine whether the effect of CRTH2 on nasal hyperreactivity is due to the control of Th2 responses or to a direct effect on mast cells.

Treatment with ramatroban, a CRTH2/TP dual antagonist, induced a reduction in several indicators of JCP such as sneezing, Cry j 1-specific IgG1 production, and Cry j 1-induced IL-4 production. It is known that ramatroban suppresses allergic responses including nasal signs both in vivo and in vitro (11, 24, 46, 48). For example, ramatroban significantly inhibited sneezing and nasal rubbing induced by Ag in actively sensitized C57BL/6 mice and guinea pigs (46, 48). Our present results are consistent with these reports and support the findings seen in CRTH2$^{-/-}$ mice that suggest a proinflammatory role of CRTH2 in allergic rhinitis. On the other hand, treatment with ramatroban was less effective than CRTH2 deficiency in all parameters of investigation. One of the possible reasons is that ramatroban antagonizes not only CRTH2 but also TP. Since it is not fully elucidated whether signals through TP, especially in mice, are proinflammatory or antiinflammatory in allergic rhinitis, simultaneous blockage with TP may affect changes of the outcomes induced by CRTH2 antagonism.

In conclusion, we developed a novel model of murine allergic rhinitis that mimics pollinosis. Additionally, we found that CRTH2 plays an essential role in the initiation of allergic rhinitis in mice. These results suggest that this murine model will be useful for elucidating the pathophysiology of allergic rhinitis, especially JCP. These observations may provide a basis for developing therapeutic approaches for managing allergic rhinitis, specifically by inhibiting PGD_2-CRTH2 interactions in the nose of individuals with allergic rhinitis.

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Disclosures

The authors have no financial conflicts of interest.

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分担研究報告書
免疫療法の効果的な投与方法と作用機序に関する研究

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研究要旨
本研究では舌下免疫療法の効果的な投与法と作用機序を解明する目的とした。成人スギ花粉症の舌下免疫療法の効果とQOLへの影響を詳細に検討し、治療期間や治療中止による影響を検討した。皮下免疫療法、薬物療法と比較し、舌下免疫療法の効果とQOLは皮下免疫療法には劣るが、薬物療法には優っていた。これらの結果はスギ花粉飛散総数にも影響された。治療期間は経年的に効果が高まり、3年以上の継続が勧められた。さらに、本邦で初めて小児舌下免疫療法を開始し、成人よりも効果の高いことを証明した。作用機序においては、誘導性制御性T細胞に着目し、IL-10産生性誘導性制御性T細胞(Tr1)が舌下免疫療法で増加することを証明した。これらの結果は今後の舌下免疫療法の発展に寄与すると考えられる。

A. 研究目的
本研究では舌下免疫療法の効果的な投与法と作用機序を解明する目的とした。成人スギ花粉症の舌下免疫療法の位置づけを皮下免疫療法や薬物療法と効果およびQOLへの影響を比較することにより評価した。また、どれくらいの期間の治療が勧められるか、治療中止による影響はどうかを検討した。さらに、本邦で初めて小児舌下免疫療法を開始した。免疫療法は皮下法をふくめて作用機序が完全に解明されておらず、IL-10産生性誘導性制御性T細胞に着目して検討した。

B. 方法
(1)舌下免疫療法の効果の検討　初期療法(薬物療法)・舌下免疫療法・皮下免疫療法の3群を設定し、少量飛散年(2010年)・中等度飛散年(2008年)と大量飛散年(2009年)の3年間で毎日の症状スコアとQOLを検討した。鼻アレルギー診療ガイドラインに従ってくしゃみ、鼻汁、鼻閉、眼の痒みの症状スコア、薬物使用量を示す薬物スコア、鼻3症状スコアの合計の総鼻スコア、症状薬物スコアを比較した。スギ花粉飛散ピーク時に日本アレルギー性鼻炎標準QOL調査票(JRQLQ No1)を用いてQOL調査を行った。2009年に治療開始後1年目(n=7)、2年目(n=18)、4年目(n=5)となる3群で比較し、継年治療の意義と効果の上乗せを検討した。2008年まで3年間治療した例で、2008年に終了した3年終了群(n=12)と2009年も治療継続した4年目群(n=5)の比較を行い、治療期間の検討を行った。

(2) 小児スギ花粉症の舌下免疫療法
2008年に小児スギ花粉症15例(男児9例、女児6例、平均8.8±2.3歳)を対象にpreliminaryに舌下免疫療法を行った。その結果と安全性のもとに2009年に61例で検討し、2010年には対照として23例の薬物療法群をおいた。小児舌下免疫療法は成人と同じ方法、同じアレルゲン量で行った。

(3)舌下免疫療法の作用機序の検討
成人舌下免疫療法例の末梢血を採取し、リンパ球を分離した。CD4陽性細胞を選択的に採取し、スギ花粉抗原刺激下に培養した。FACSにより制御性T細胞およびIL-10産生性誘導性制御性T細胞(Tr1)を検討した。また、proliferation assayでスギ花粉主要抗原またはCD3/CD28刺激による細胞分化能を検討した。さらに、抗IL-10および抗IL-10受容体で中和した場合のproliferation assayも行った。次に、治療効果の高かった小児例で、IL-10産生性誘導性制御性T細胞(Tr1)と単球からのIL-10産生能を前向きに検討した。また、血清IgG4, IL-17A, IL-31, IL-33をELISAで測定した。

C. 結果
(1) 成人舌下免疫療法の効果と位置づけ　舌下免疫療法は、花粉飛散とともに症状が出現し、皮下免疫療法より劣っていたが、初期療法より有意に良好であった。但し、薬物スコアは皮下法同様に低く良好で、効果があり薬物使用を少なくできる治療と考えられた。QOL調査も同様に皮下法に

は劣るが薬物療法より良好であった。これらの結果は花粉飛散数に影響を受け、大量飛散年には3群の差が縮まり、少量飛散ではすべての治療で有効なため差が無くなった。継年治療の意義として、症状スコアと薬物スコアともに治療年数が増すと効果が増強していており、治療を3年で終了せずに4年目も行う方がよいと考えられた。

(2) 小児舌下免疫療法　小児でも成人と同じ投与方法で安全に在宅投与ができ、効果が高いことが確認された。大量飛散年の2009年でも症状スコアは最大でくしゃみ1.3点、鼻汁1.5点、鼻閉1.6点、眼の痒み1.7点であった。また、薬物スコアは飛散ピーク時でも1点未満であり、61 例中 19 例(32%)が無投薬であった。アンケートで全体の80%で効果が認められた。スコアでは、成人舌下免疫療法より効果が高く、成人皮下免疫療法と同等以上の効果があった。対照例をおいた2010年は少量飛散のため、両治療ともに症状が少なく、差はなかった。引き続きの検討を予定した。

(3) 作用機序の検討　CD4陽性細胞に占める制御性 T リンパ球の割合は舌下免疫療法で natural regulatory T cell は増加しないが、IL-10産生性誘導性制御性T細胞(Tr1)は増加していた。健常人で高いTr1は、未治療スギ花粉症例で低く、免疫療法療法で健常人と同等であった。スギ抗原刺激だけでなく、抗原非特異的なCD3/CD28刺激でもTr1が増加した。また、抗IL-10 および抗IL-10受容体の中和で、細胞分化能は強くなっており、IL-10 が functional であることが示された。小児でもIL-10産生性T細胞とIL-10産生性CD14陽性単球の経時的増加があった。IgG4は全体的には経時的な増加に乏しかったが、症状薬物スコアで効果良好群と不良群に分類すると、良好群で IgG4の増加傾向があった。IL-17A, IL-31, IL-33に一定の傾向がなかったが、小児の IL-31 は高かった。

D. 考察

スギ花粉症の舌下免疫療法の期待が高まっている。欧州ではアレルギー性鼻炎の舌下免疫療法が広まっているが、スギ花粉は本邦に特有のアレルゲンであり、本邦独自での検討が必要である。今回、これまでにおこなってきた皮下免疫療法の知見をもとに成人舌下免疫療法の効果と継続治療の意義を検討した。その結果、薬物量を少なくし、初期治療よりも効果の高い治療であると考えられ、簡易で安全に行える有用性の高い治療と考えた。また、経年治療による効果が証明され、今後の検討も必要であるが治療の方向性が導けたと考えられる。小児の舌下免疫療法でより効果が高かったことは大きな収穫であった。皮下注射法と同等以上の効果を得たことは、成人舌下免疫療法での効果不足の欠点を考察する大きなヒントとなるかもしれない。小児舌下免疫療法の抗原投与量は体重あたりに換算すると成人の2ー3倍にあたるため、成人の抗原投与量を増加すればもっと効果が期待できるかもしれない。

今回、作用機序の解明に大きな展開が得られたと考える。舌下免疫療法にTr1が関与する可能性が考えられ、T 細胞分化能から IL-10 がかかわっていることもわかっただけでなく、非特異的刺激である CD3/28 刺激でも Tr1 が誘導できたことは、スギ花粉症の免疫療法がスギ花粉抗原以外のアレルギー性鼻炎にも有効的に働く可能性が示唆された。

E. 結論

本研究で舌下免疫療法の位置づけがさらにはっきりし、治療の方向性が明確となったとともに、作用機序への足がかりができたと考える。

F. 健康危険情報

該当事項なし

G. 研究発表

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H. 知的財産権の出願・登録状況

なし

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原　著

スギ花粉症に対する舌下免疫療法の治療年数による臨床効果の増強と治療終了後の継続効果

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【目的】スギ花粉症の舌下免疫療法が期待されるが，検討すべき課題も多い．舌下免疫療法での治療継続による臨床効果増強と必要治療年数を検討した．
【方法】舌下免疫療法を開始して1年目（n=7），2年目（n=18），4年目（n=5）の3群で，2009年のスギ花粉飛散期（大量飛散年）の臨床効果を比較した．また，舌下免疫を2008年まで3年間行い，2009年も行った例（n=5）と行わなかった例（n=12）を比較し，3年で終了できるかを検討した．
【結果】治療年数が増すに従い，くしゃみ，鼻汁，鼻閉の症状スコアが全般に良くなり，症状薬物スコアが有意に4年目で良好であった．4年継続例は3年終了例より症状薬物スコアで有意に良好であった．
【結論】舌下免疫療法での治療年数による効果増強が認められた．また，治療を3年で終了せずに4年目も継続する方が良いと考えられたが，検討例数が少なく今後の検討も必要である．

Key words: Japanese cedar —— pollinosis —— sublingual immunotherapy

スギ花粉症の有病率は急増しており[1)]，有効な治療が望まれている．鼻アレルギー診療ガイドライン（2009年版）[2)]による重症度に応じた花粉症治療が示され，内服薬による初期療法の有用性が示されている．また，皮下注射による免疫療法（皮下免疫療法）も推奨され，薬物療法よりも高い有効性が報告されている[3)-6)]．急増するスギ花粉症においてより多くの患者に皮下免疫療法を行えれば理想的であるが，注射法であるため痛みがあること，治療開始後6カ月程度は頻回の通院が必要であること，稀ながらアナフィラキシーを含む副反応があること，などの皮下免疫療法自体の有する問題点も多く，広く普及するには至らない．舌下免疫療法は皮下免疫療法の問題点を解決できる新しい治療法として期待されている．本邦では舌下免疫療法の保険適応はないが，少数の施設でスギ花粉症に対して臨床研究が試みられている．我々は，成人スギ花粉症に対する舌下免疫療法を2005年末より行っており，これまでの結果から免疫療法の効果に関しては，皮下免疫療法には及ばないが初期療法より高く，特に併用薬剤を大きく減らすことが可能であると報告してきた[7)8)]．

皮下免疫療法では，治療開始直後よりも数年間の治療継続により効果が増強する[9)]．また，3年か

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Abbreviation: SLIT "sublingual immunotherapy"
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Table 1 Profile of subjects

	1st year	2nd year	4th year	Finished at 3rd year	*P* value
Male/Female	1/6	8/10	4/1	8/4	ns
Age	29.4 ± 14.5	45.8 ± 15.4	52.6 ± 9.8	46.2 ± 13.6	ns
Japanese cedar CAP score	4.7 ± 1.1	4.1 ± 1.1	4.1 ± 1.0	3.5 ± 1.0	ns

Values represent mean with standard deviation: mean ± SD. *P* value was calculated by chi-square test for sex or Mann-Whitney U test for age and Cap score.

ら5年間の治療を継続すると，治療を中止しても効果が長期間持続すると報告されている[10)-12)]．一方で，スギ花粉症の舌下免疫療法は臨床検討段階であり，皮下免疫療法同様の継年治療による効果の上乗せがあるか，何年間の治療期間が必要かなどは不明である．舌下免疫療法に必要なアレルゲン量は皮下免疫療法よりも大量であることを考えれば，より短期間の治療年数で終了し，且つ，効果が長期間に持続できれば，医療経済面からも適切と考えられる．

そこで，当科で舌下免疫療法を継続している例で継年治療による臨床効果の上乗せがあるかを検討した．また，3年間で治療を終了した例の追跡調査を行い，3年で治療を終了できるかも検討した．

対象と方法

対象は，三重大学医学部倫理委員会承認のもとに中等症以上の成人スギ花粉症にスギ花粉抗原特異的舌下免疫療法を行った例である．舌下免疫療法の治療適応は皮下免疫療法の適応と同じで，世界保健機構の見解書[12)]に示される皮下免疫療法の適応に準じた．舌下免疫療法の方法は既報[13)]の通りであるが，標準化アレルゲン治療エキス「スギ花粉」(現在，標準化アレルゲン治療エキス皮下皮内注射「スギ花粉」)を用い，アレルゲン希釈液で2JAU/mlに希釈した治療液で12月初旬より開始した．毎日増量しながら舌下に2分間投与後に吐き出し(舌下吐き出し法)，4週間で2000JAUまで増量した．維持期は週1回で2000JAUを舌下投与し，花粉飛散の終了とともに舌下投与も終了して，翌12月より同じスケジュールで繰り返した．

舌下免疫療法は，全例12月初旬に開始しているため，治療期間により以下の4群に分類した．

1) 1年目群：2008年12月より治療を開始し，2009年に治療開始後1シーズン目を迎えた7例

2) 2年目群：2007年12月より治療を開始し，2009年に治療開始後2シーズン目を迎えた18例

3) 4年目群：2005年12月より治療を開始し，2009年に治療開始後4シーズン目を迎えた5例

4) 3年終了群：2005年12月より治療を開始し，3年間の継続治療の後に終了し，4年目は舌下免疫療法を行わず，症状に応じて薬物治療を行った12例．

なお，舌下免疫療法は臨床研究として開始し，2006年に新規患者を募集しなかったため3年目群はなかった．

各群の背景をTable 1に示す．年齢，性差，CAP法による血清スギ特異的IgE抗体に有意な差がなかった．

レスキュー薬

舌下免疫療法を行っても症状が出現する可能性があるため，症状に応じた投薬治療を行った．1年目群，2年目群，4年目群には，舌下免疫療法の効果を確認するために初期療法を行わなかったが，飛散開始後の治療については鼻アレルギー診療ガイドライン（2009年版)[2)]を参考にして，薬剤の種類および使用量に制限を加えなかった．ただし，内服ステロイドは舌下免疫療法の免疫応答に関与するため禁止とした．局所ステロイドの使用は可能とした．3年終了群も同様に治療に制限をしなかった．

臨床効果判定

花粉飛散がみこまれる2月と3月の毎日の症状と使用した薬剤を各自が症状日記に記載した．くしゃみ，鼻汁，鼻閉の鼻3症状は鼻アレルギー診療ガイドライン(2009年版)[2)]の記載に準じてスコア化し，症状スコア（symptom score）とした（Table 2）．使用薬剤

Table 2 Nasal symptom score

Score	4	3	2	1	0
Number of sneezing (times)	≧21	11-20	6-10	1-5	None
Number of nasal blowing (times)	≧21	11-20	6-10	1-5	None
Congestion	Complete, all day	Very severe, with frequent oral-breathing	Severe, with occasional oral-breathing	(+) without oral-breathing	None

Nasal symptom score of each subject was based on Practical Guideline for the Management of Allergic Rhinitis in Japan[2].

Table 3 Medication score

Medication score	
antihistamine	
oral	1 point
eye drop	1 point
steroid	
nasal	2 points
eye drop	2 points
vasoconstriction	
nasal	1 point

Medication score of oral steroid was calculated with reference to predonisolone (1 point/1 mg).

Medication score was based on Practical Guideline for the Management of Allergic Rhinitis in Japan[2].

も同ガイドラインに準じて薬物スコア（medication score）とした（Table 3）．皮下免疫療法の維持期には薬物スコア1点が付加されるが，舌下免疫療法の薬物スコアの基準はなく，本研究が舌下免疫療法間の比較であるので今回は舌下免疫療法自体に薬物スコアを設定しなかった．鼻3症状の症状スコアの合計を総鼻症状スコア（total nasal symptom score），鼻3症状の症状スコアのなかの最大スコアと薬物スコアの合計を症状薬物スコア（symptom-medication score）とした．

花粉飛散数測定

診療施設内（三重県津市）の9階建てビル屋上に設置したダーラム型花粉収集器で補足した毎日のスギ花粉を計測し，2009年の1日毎のスギ花粉飛散動態を観察した．2009年のスギ花粉飛散総数は11,941個/cm^2で，過去26年間の中央値（2,808個/cm^2）の約4倍となる大量飛散年となった．スギ花粉飛散動態は結果のグラフ上に併記した．

解析

1日毎の各群間の解析には，Bonferroni/Dunn検定を用い，$p<0.05$をもって有意差ありとした．

結　果

治療年数による臨床効果（Fig. 1a-f）

治療年数が増えると臨床効果も良くなるかを検討する目的で，1年目群，2年目群，4年目群を比較した．鼻汁スコア（Fig. 1b）は，花粉飛散とともに多数日で4年目群が1年目群や2年目群より有意に良好であった．鼻閉スコア（Fig. 1c）は，2月前半で1年目群の症状が有意に高いが，花粉非飛散期であることから治療効果による違いとは考えにくかった．総鼻症状（Fig. 1d）では，全般に治療年数が増すと効果が高い傾向にあり，4年目群は1年目群より多数日で有意に良好であった．薬物スコア（Fig. 1e）も有意では無いが同様の傾向にあった．症状薬物スコア（Fig. 1f）は3群で有意差のある日が多くなり，舌下免疫療法は治療年数が増えると効果が高くなるといえる．

治療終了の影響

舌下免疫療法を3年で終了しても翌年に効果が持続しているかを検討する目的で，4年目群と3年終了群を比較した．両群は2006年から2008年までの3年間に舌下免疫療法を行い，4年目も継続した4年目群と4年目は舌下免疫療法を行わな