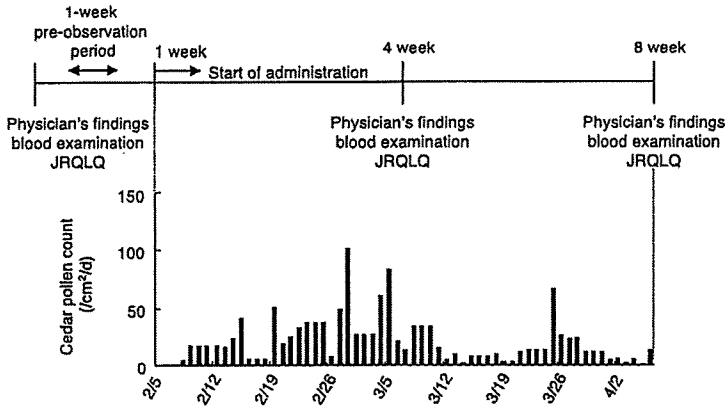

Fig. 1. Study Protocol and Diagrammatic Profile of Japanese Cedar Pollen Dispersion during the Clinical Trial in Tokyo, in 2007.
These data were obtained from the Bureau of Social Welfare and Public Health, Tokyo Metropolitan Government, by determining the pollen collected in a Durham pollen catcher on the roof of a building in Chiyoda Ward.

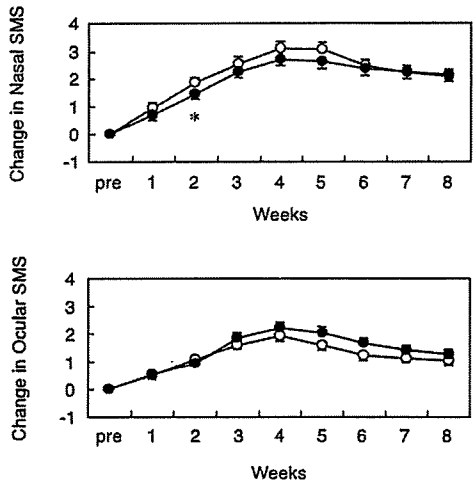

Fig. 2. Time-Course Change in Nasal and Ocular Symptom Medication Scores (SMS).
The scores are differences from those in the pre-observation period. Placebo group (O), $n = 53$; OLL2809 group (●), $n = 47$. $^*p < 0.05$ (Mann-Whitney's U test).

(Fig. 1), according to a survey conducted by the Bureau of Social Welfare and Public Health of the Tokyo Metropolitan Government.

Analysis of subjects

The nasal cavity findings, blood examinations, and JRQLQ results revealed no significant differences between the placebo and OLL2809 groups that were administered *L. gasseri* OLL2809. Examination of the allergy diaries revealed that although ocular SMS did not differ between the groups, and nasal SMS at 2 weeks was significantly low ($p = 0.0409$) in the OLL2809 group as compared with the placebo group (Fig. 2). Overall, no efficacy of *L. gasseri* OLL2809 was observed in this study.

Subgroup analysis: analysis of high CAP-RAST score group

Type I allergic diseases are characterized by an elevation in serum antigen-specific IgE levels.[21] The CAP-RAST score of the subjects enrolled in this study was 3.22 ± 0.80 (placebo group, 3.28 ± 0.77, $n = 53$; OLL2809 group, 3.15 ± 0.83, $n = 47$; $p = 0.3371$). Hence, each group was divided into two subgroups. The first subgroup consisted of subjects with CAP-RAST scores of 2 or 3, and the second group of subjects with CAP-RAST scores of 4 or 5. Subsequently, these subgroups were analyzed again to investigate the efficacy of *L. gasseri* OLL2809 on them. Although there were no significant differences between the placebo and OLL2809 subgroups with CAP-RAST scores of 2 or 3 (data not shown), *L. gasseri* OLL2809 was found to be effective, particularly in subgroups of subjects with CAP-RAST scores of 4 or 5, as described below.

Background characteristics of subjects with CAP-RAST scores of 4 or 5

The baseline characteristics of the subjects were similar in the placebo and OLL2809 subgroups, with CAP-RAST scores of 4 or 5, as well as those of the all subjects, in terms of age, sex, duration of JCP, nasal cavity findings, nasal and ocular symptom medication scores taken in allergy diaries and blood exams (Table 1).

Nasal cavity findings

Both the nasal cavity scores for mucosal swelling of the inferior turbinate and the amount of watery rhinorrhea significantly ($p < 0.01$) increased at 4 weeks in both the placebo and OLL2809 subgroups. However, no differences were observed between these subgroups. When the scores were summed and total scores were compared, the results tended to be low ($p = 0.0991$) in the OLL2809 subgroup at 4 weeks (Table 2). The scores decreased at 8 weeks as compared to those at 4 weeks, and no difference was observed between the subgroups.

Nasal and ocular SMS

Both nasal and ocular SMS increased and symptoms were exacerbated, with a peak at 4 to 5 weeks during the experimental period. While no differences were observed in the ocular SMS between the subgroups, the nasal SMS exhibited significantly ($p < 0.05$) lower values in the OLL2809 subgroup than in the placebo subgroup at 1, 5, 6, 7 and 8 weeks (Fig. 3).

JRQLQ

The scores for all the QOL items in the JRQLQ-I increased at 4 weeks, and then slightly decreased at 8

Table 1. Background Factors of Subjects with CAP-RAST Scores of 4 or 5

	Placebo ($n = 19$)	OLL2809 ($n = 12$)	p-value
Age (year)	30.3 ± 1.6	30.7 ± 2.1	0.8766[a]
Sex (male:female)	8:11	6:6	0.9524[b]
Duration of JCP (year)	10.3 ± 1.6	9.9 ± 1.4	0.8503[a]
Mucosal swelling of the inferior turbinate	0.474 ± 0.177	0.750 ± 0.250	0.3273[c]
Amount of watery rhinorrhea	0.211 ± 0.096	0.167 ± 0.112	0.7671[c]
Total nasal finding scores	0.684 ± 0.242	0.971 ± 0.336	0.5015[c]
Ocular symptom medication score	0.233 ± 0.085	0.036 ± 0.020	0.1066[c]
Nasal symptom medication score	1.02 ± 0.27	1.17 ± 0.31	0.6245[c]
Total-IgE (IU/ml)	281 ± 60	296 ± 87	0.8845[a]
Japanese cedar pollen-specific IgE (UA/ml)	38.7 ± 4.3	42.6 ± 6.0	0.5887[a]
Eosinophils (%)	2.58 ± 0.44	2.58 ± 0.34	0.9937[a]
Th1/Th2 ratio	8.16 ± 0.60	8.77 ± 1.36	0.6486[a]

[a,b,c]Analyzed by Student's t-test, Chi-square test, and Mann-Whitney's U test respectively.
Th1/Th2 ratio represents the proportion of IFN-γ^+CD4$^+$ and IL-4$^+$CD4$^+$ peripheral mononuclear cells.

Table 2. Mean Change in Scores for Nasal Cavity Findings for Subjects with CAP-RAST Scores of 4 or 5 after 4 and 8 Weeks of Treatment

	4 weeks			8 weeks		
	Placebo	OLL2809	p-value	Placebo	OLL2809	p-value
Mucosal swelling of the inferior turbinate	0.842 ± 0.206	0.500 ± 0.230	0.3837	0.474 ± 0.160	0.417 ± 0.149	0.8018
Amount of watery rhinorrhea	0.895 ± 0.130	0.667 ± 0.142	0.2766	0.211 ± 0.123	0.500 ± 0.195	0.1411
Total nasal finding scores	1.737 ± 0.295	1.167 ± 0.271	0.0991	0.684 ± 0.242	0.917 ± 0.260	0.2478

The scores are differences from those in the pre-observation period. Total nasal finding scores represent sums of the scores for swelling of the inferior turbinate and amount of watery rhinorrhea. Placebo subgroup, $n = 19$; OLL2809 subgroup, $n = 12$.

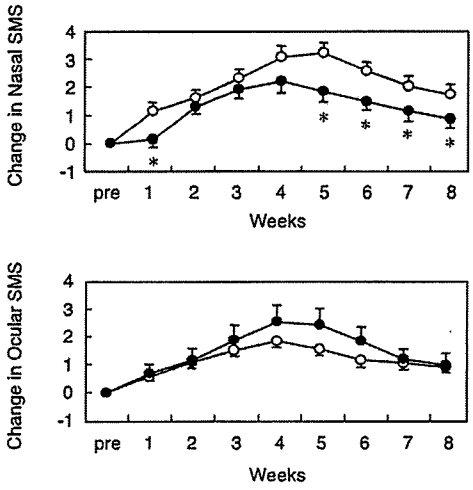

Fig. 3. Time-Course Change in Nasal and Ocular Symptom Medication Scores (SMS) of Subgroups with CAP-RAST Scores of 4 or 5.
The scores are differences from those in the pre-observation period. Placebo subgroup (○), $n = 19$; OLL2809 subgroup (●), $n = 12$. $^*p < 0.05$ (Mann-Whitney's U test).

weeks. Although there was no significant difference in the scores between the subgroups at 4 weeks (data not shown), the scores for nasal congestion and itchy nose were significantly lower ($p = 0.009$ and 0.0156 respectively), and the frequency of the subjects who exhibited no symptoms was higher in the OLL2809 subgroup at 8 weeks (Fig. 4).

Of the 17 items in JRQLQ-II, the scores for reduced memory, reduced contact with friends or others by telephone or conversation at 4 weeks and for tiredness at 8 weeks tended to be low ($p = 0.0666$, 0.0934, and 0.0848 respectively) in the OLL2809 subgroup (Table 3). Furthermore, of the 17 items, scores at lower values were observed in 15 items in the OLL2809 subgroup at 8 weeks. Although the subjects responded to the questionnaires at 4 and 8 weeks, they were queried regarding the severity of their symptoms during the 1–2 weeks preceding the time of response. Therefore, these scores at 8 weeks represent symptoms occurring at 6 to 7 weeks, and they correspond well with the nasal SMS recorded in the allergy diaries.

Blood examination

At 4 weeks, all the blood examination items, including the total IgE and Japanese cedar pollen-specific IgE levels, numbers of eosinophils, and the Th1/Th2 ratio, increased, with a peak at 4 weeks, but no differences were observed between the placebo and OLL2809 subgroups. When we analyzed the relative values where the mean values in each subgroup at the pre-observation period were expressed as 1.0, the Japanese cedar pollen-specific IgE levels in the OLL2809 subgroup tended to be lower than in the placebo subgroup at 4 weeks ($p = 0.0525$, Fig. 5). While there were no intra-subgroup differences even in the relative values of eosinophils, intra-period analysis in each subgroup revealed that although this value significantly ($p < 0.05$) increased in the placebo subgroup at 8 weeks as compared with the pre-observation period, this increase was not significant in the OLL2809 subgroup ($p = 0.2104$). Further, the Th1/Th2 ratio tended to increase in the OLL2809 subgroup but not in the placebo subgroup as compared with the respective values for the pre-observation period ($p = 0.0687$). Total IgE did not significantly change between the subgroups during the study period.

Safety

No adverse effects were observed throughout the study. No significant changes in blood or biochemistry

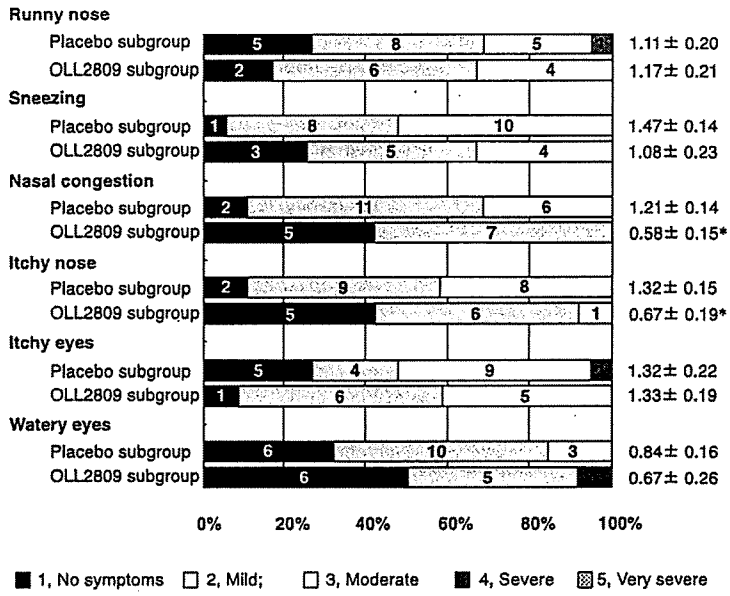

Fig. 4. Japanese Allergic Rhinitis Standard Quality of Life Questionnaire for Subgroups with CAP-RAST Scores of 4 or 5 after 8 Weeks of Treatment.

The numbers in the histogram represent the numbers of subjects, and the mean scores ± SE are presented on the right. Placebo subgroup, $n = 19$; OLL2809 subgroup, $n = 12$. $^*p < 0.05$ (Mann-Whitney's U test).

Table 3. Mean Change in JRQLQ-II Scores of Subgroups with CAP-RAST Scores of 4 or 5 after 4 and 8 Weeks of Treatment

	4 weeks			8 weeks		
	Placebo	OLL2809	*p*-value	Placebo	OLL2809	*p*-value
1. Reduced productivity at work/home	1.158 ± 0.220	0.667 ± 0.225	0.2142	0.632 ± 0.191	0.333 ± 0.188	0.3555
2. Poor mental concentration	1.158 ± 0.206	0.833 ± 0.271	0.4170	0.632 ± 0.157	0.583 ± 0.229	>0.999
3. Reduced thinking power	1.053 ± 0.223	0.833 ± 0.271	0.6399	0.632 ± 0.175	0.417 ± 0.229	0.5180
4. Impaired reading book/newspaper	0.842 ± 0.206	0.417 ± 0.193	0.5083	0.526 ± 0.160	0.333 ± 0.142	0.5083
5. Reduced memory	0.789 ± 0.224	0.167 ± 0.167	0.0666	0.316 ± 0.134	0.083 ± 0.149	0.3137
6. Limitation of outdoor life	0.842 ± 0.175	0.417 ± 0.228	0.1014	0.579 ± 0.221	0.333 ± 0.225	0.6341
7. Limitation of going out	1.053 ± 0.235	0.750 ± 0.279	0.4114	0.579 ± 0.176	0.417 ± 0.193	0.5745
8. Hesitation visiting friend or relatives	0.789 ± 0.196	0.417 ± 0.260	0.4558	0.421 ± 0.139	0.250 ± 0.131	0.4558
9. Reduced contact with friends or others by telephone or conversation	0.632 ± 0.175	0.250 ± 0.131	0.0934	0.316 ± 0.134	0.250 ± 0.131	0.8731
10. Not an easy person to be around	0.632 ± 0.191	0.250 ± 0.131	0.8672	0.263 ± 0.129	0.250 ± 0.131	0.8672
11. Impaired sleeping	0.421 ± 0.221	0.750 ± 0.372	0.4932	0.316 ± 0.134	0.333 ± 0.142	0.7584
12. Tiredness	1.000 ± 0.229	1.000 ± 0.348	0.8301	0.737 ± 0.185	0.250 ± 0.131	0.0848
13. Fatigue	0.789 ± 0.211	1.083 ± 0.358	0.5735	0.579 ± 0.221	0.333 ± 0.142	0.5817
14. Frustration	0.789 ± 0.224	0.917 ± 0.358	0.9828	0.316 ± 0.154	0.417 ± 0.149	0.5548
15. Irritability	0.842 ± 0.191	1.000 ± 0.326	0.8251	0.368 ± 0.114	0.250 ± 0.131	0.4991
16. Depression	0.737 ± 0.200	0.833 ± 0.366	0.8419	0.263 ± 0.129	0.250 ± 0.131	0.8814
17. Unhappiness	0.759 ± 0.192	0.833 ± 0.366	0.8423	0.211 ± 0.164	0.333 ± 0.142	0.7151

The scores are differences from those in the pre-observation period.
Placebo subgroup, $n = 19$; OLL2809 subgroup, $n = 12$.

results were observed during the study period in any of the subjects.

Discussion

In recent years, a number of clinical trials have evaluated the efficacy of probiotics and of heat-killed lactobacilli in allergic diseases. The results imply that some clinical effects on pollinosis,[22–25] atopic dermatitis,[26,27] perennial allergic rhinitis induced by house-dust mites,[28] and food allergy[29] occurred. On the other hand, Brouwer *et al.* and Grüber *et al.* reported that there were no clinical or immunological effects of probiotic *L. rhamnosus* GG, which was used in infants with atopic dermatitis.[30,31] Such diverse results were due to differences in study design, and perhaps were due to heterogeneity of exposure to the allergens, the allergic backgrounds of the subjects, and the efficacy of the microorganisms used. Hence, further studies are required to determine the efficacy of probiotics and of lactic acid bacteria with immunoregulatory activity.

Although our clinical study had a substantial sample size, no obvious efficacy of *L. gasseri* OLL2809 was observed. Hence, we performed subgroup analyses based on the CAP-RAST scores. Because serum antigen-specific IgE levels play a crucial role in the

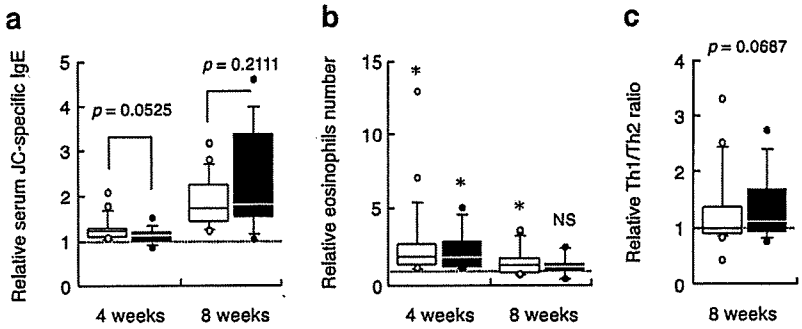

Fig. 5. Relative Changes in Serum Japanese Cedar Pollen-Specific IgE (a), Number of Peripheral Blood Eosinophils (b), and the Th1/Th2 Ratio (c) in Subgroups of Subjects with CAP-RAST Scores of 4 or 5.

Data are represented by their relative values where the mean values in each subgroup at the pre-observation period are expressed as 1.0 (broken line). The differences in the relative serum Japanese cedar pollen-specific IgE between the placebo and OLL2809 subgroups were analyzed by Student's *t*-test. Those in the relative eosinophil numbers and relative Th1/Th2 ratio between pre-observation and 4 and 8 weeks after treatment were analyzed in each group by Student's paired *t*-test with Bonferroni's correction (*$p < 0.05$). NS, not significant. Placebo subgroup (open bars), $n = 19$; OLL2809 subgroup (solid bars), $n = 12$.

onset of symptoms in type I allergic diseases, it is possible that subjects with high CAP-RAST scores have a higher predisposition to allergic diseases. The results of the subgroup analyses revealed that subjects with CAP-RAST scores of 4 or 5 exhibited the efficacy of *L. gasseri* OLL2809: it caused a significant reduction in the nasal SMS throughout the administration period, and the nasal symptoms in JRQLQ at 8 weeks. In addition, the relevant evaluation items such as clinical scores for the nasal cavity at 4 weeks and the serum allergy-related items, including the Japanese cedar pollen-specific IgE levels, eosinophils, and the Th1/Th2 ratio improved, though the differences were not statistically significant. There was a difference in that efficacies were observed for each item, *e.g.*, the nasal cavity findings tended to be low in the OLL2809 subgroup at 4 weeks, but other parameters such as nasal SMS, nasal congestion, and itchy nose in JRQLQ-I were lower in the OLL2809 subgroup in the later period (5–8 weeks). This might have been caused by a difference in objective and subjective evaluation. For instance, even if an objective examination item is improved, subjective symptoms are not necessarily improved, and *vice versa*. This sometimes occurs in clinical examination. Yet, considering these data comprehensively, it was assumed that *L. gasseri* OLL2809 mainly ameliorated the nasal symptoms of the subjects with CAP-RAST scores of 4 or 5, *via* affecting the immune systems.

Similar results have been reported for *L. casei* strain Shirota. Tamura *et al.* reported that supplementation with *L. casei* strain Shirota did not affected the nasal or ocular SMS, but it tended to reduce the nasal SMS in the subgroup of subjects with moderate to severe nasal symptom scores prior to start of ingestion of the test samples.[25] Type I allergic diseases such as JCP are associated with elevated serum antigen-specific IgE. Binding of inhaled allergens to IgE on the surfaces of basophils and mast cells, with subsequent cross-linkage of IgE and aggregation of high-affinity receptors for IgE (FcεRI), triggers the release of histamine, leukotrienes, and other inflammatory mediators, followed by the onset of allergic symptoms.[32] Consequently, there must be a correlation between the symptoms and the Japanese cedar pollen-specific IgE levels.

Here, we hypothesize the mechanism by which *L. gasseri* OLL2809 exhibited efficacy in the subjects with CAP-RAST scores of 4 or 5. It has been widely reported that some *Lactobacillus* strains, such as *L. gasseri* OLL2809, stimulate IL-12 (p70) production by immune competent cells, and that this promotes a shift in the Th1/Th2 balance from Th2 toward Th1.[16–18] This immunoregulatory effect in the Th1/Th2 balance is observed as certain lactobacilli induce IFN-γ production and reduce IL-4 production by $CD4^+$ T cells.[16,33] However, this effect occurs when antigen-sensitized $CD4^+$ T cells are stimulated by antigens. For instance, when $CD4^+$ transgenic T cells expressing ovalbumin-specific T-cell receptors were cultured with *Mycobacterium tuberculosis* in the absence of ovalbumin, they did not produce substantial levels of IFN-γ or IL-4 as compared with those cultured in the presence of both *M. tuberculosis* and ovalbumin.[34] Likewise, stimulation of IFN-γ production and suppression of IL-4 production by *L. gasseri* OLL2809 are observed specifically in antigen-sensitized $CD4^+$ T cells when the antigen is present in the cell culture, whereas it does not occur in non-sensitized $CD4^+$ T cells even in the presence of the antigen (Sashihara *et al.*, unpublished observation). This suggests that the immunostimulatory effect of microbes in shifting the Th1/Th2 balance from Th2 to Th1 can be effective when the host immune cells are highly sensitized and the antigen level is sufficient to stimulate antigen-sensitized $CD4^+$ T cells. In the present clinical trial, it is hard to assume that there was a difference in the amount of cedar pollen exposed in the subjects. Therefore, the observation that *L. gasseri* OLL2809 was effective in the subjects with CAP-RAST scores of 4 or 5 suggests that their $CD4^+$ T cells were highly sensitized to the antigen, and that consequently the reactivity of the immune cells to *L. gasseri* OLL2809 was higher than those from subjects with CAP-RAST scores of 2 or 3.

In conclusion, although no obvious clinical efficacy of heat-killed *L. gasseri* OLL2809 was observed in subjects with JCP, this strain possesses efficacy to ameliorate symptoms by modulating the systemic immune responses in subjects with a high predisposition to allergic disease.

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Validation Study of the OHIO Chamber in Patients with Japanese Cedar Pollinosis

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Key Words
Allergen exposure · Clinical trial · Japanese cedar pollen · Japanese patients · OHIO Chamber · Seasonal allergic rhinitis · Validation study

Abstract
Background: An artificial exposure chamber (OHIO Chamber), which allows dispersal of a fixed concentration of Japanese cedar (JC) pollen under stable conditions, was constructed. This study was conducted to identify the exposure conditions assuring validity of the clinical tests conducted using this chamber. ***Methods:*** Twenty-four adult patients with JC pollinosis were exposed to different concentrations of JC pollen: 0 (only during the summer period), 4,000, 8,000 and 12,000 grains/m^3, and the nasal and ocular symptoms were self-assessed during a 4-hour period of exposure. The amount of nasal discharge was measured and the sneezing frequency was recorded. This study was conducted twice during the summer and winter periods, i.e. non-pollen seasons. The reproducibility of the symptoms between the two seasons was assessed. ***Results:*** None of the subjects developed any symptom at the pollen concentration of 0 grains/m^3. No significant differences in the time to the onset of symptoms were found between the summer and winter study, regardless of the pollen concentration. There were no significant differences between the summer and winter study in the total symptom score and total nasal symptom score at any pollen concentration, suggesting the very favorable reproducibility of symptoms. ***Conclusions:*** Efficient and reproducible results are obtained in patients exposed to JC pollen in the OHIO Chamber. The results suggest the conditions of JC pollen exposure have scientific validity and the OHIO Chamber has the potential to contribute significantly to basic and clinical studies of JC pollinosis.

Introduction

Japanese cedar (JC) pollinosis is a representative disease of seasonal allergic rhinitis in Japan. The incidence of JC pollinosis has steadily increased over the last 30 years, and currently the disease has been reported to occur at a prevalence of >16% in the Japanese population [1]. The age at onset of JC pollinosis is declining, and ap-

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parently the symptoms also increase in severity annually. Thus, JC pollinosis can undermine the quality of life, interfere with the activities of daily living and cause a decline in labor productivity, thus posing an important socioeconomic problem [2].

A large number of antiallergic drugs exerting different mechanisms of action have been developed and used for the treatment of JC pollinosis in the clinical setting. There have been several clinical studies on the clinical effects and safety of various drugs administered during the pollen season, i.e. between February and April [3–5]. However, the number of pollen grains dispersed varies each year and among districts, and the temperature and climate are also not constant, making it difficult to reliably compare the results of evaluations of the efficacy and safety of drugs between different years. To overcome these drawbacks, an allergen exposure unit that allows exposure to a fixed number of pollen grains in a stable environment has increasingly been employed for such studies. The use of such a chamber allows studies to be conducted under the same climatic conditions as those prevalent during the pollen season at any time of the year, which is particularly useful for the study of seasonal allergic rhinitis.

There are several allergen exposure facilities in Europe, the US and other countries, e.g. the Vienna Challenge Chamber in Austria [6], the Environmental Exposure Unit in Canada [7, 8] and Germany [9], and the allergen exposure unit in the US [10]. Clinical efficacies and onset and duration of action of antiallergic drugs have been investigated in patients with allergic rhinitis at each facility.

Owing to the large number of patients with JC pollinosis in Japan, the need was felt for the development of such an environmental exposure unit for studying JC pollinosis. Therefore, we established an environmental exposure unit (OHIO Chamber) designed to allow dispersal of JC pollen in Tokyo [11], which is the third unit established in this country after those in Wakayama [12] and Osaka [13].

It is considered necessary to establish some exposure conditions and the scientific validity necessary for the judgment of drug efficacy in such a chamber. A consensus report on the Chamber, which has recently been issued, has also shown the importance of a validation study [14]. Since there are few data concerning the conditions of exposure to JC pollen and it is difficult to compare the respective exposure chambers, we conducted a basic and systematic evaluation of the OHIO Chamber.

In the present study, the reactivity and safety of several JC pollen exposure levels were investigated in the patients during the summer and winter periods, i.e. non-pollen seasons. The results established the validity of such testing conducted in the OHIO Chamber.

Patients and Methods

Patients

Adult patients with JC pollinosis were studied. In these patients, the severity of rhinitis could be judged from diaries recording symptoms in the pollen dispersal season.

The inclusion criteria were as follows: a history of symptoms of JC pollinosis for at least 2 years, a positive RAST for JC pollen antigen (CAP-RAST class ≥ 2) and a positive provocation test with a JC pollen disc, which is a paper disc measuring 5 mm in diameter containing a defined amount of JC pollen extract (kindly supplied by Sagamihara Hospital).

The exclusion criteria were as follows: a history of nasal and/or ocular diseases prior to entry into the Chamber, a history of treatment with steroid injections within 6 months prior to entry into the Chamber, a history of treatment with oral, inhalational or topical steroids and/or antihistamines within 4 weeks prior to entry into the Chamber, evidence of upper and/or lower respiratory tract inflammation within 2 weeks prior to entry into the Chamber, asthma, or a past history of anaphylaxis; pregnant, possibly pregnant and lactating women as well as women who intend to become pregnant within the proposed study period, and patients judged to be unsuitable for participation in the study for any reason by the physicians in charge of the study.

The study was conducted in accordance with Good Clinical Practice Guidelines and the Declaration of Helsinki. It was performed during the summer and winter periods of 2006, after it was reviewed and granted prior approval by the institutional review board of the Shinanozaka Clinic. Written informed consent was obtained from all of the participants prior to their entry into the study.

Study Design

This study was a randomized, double-blind, cross-over trial. It was conducted twice: during the summer (July) and during the winter (November), i.e. non-pollen seasons.

With regard to the target pollen dispersal concentrations in the Chamber, four different concentrations were set: none (target pollen exposure level, 0 grain/m^3; only in summer), and low (4,000 grains/m^3), moderate (8,000 grains/m^3) and high concentrations (12,000 grains/m^3). The patients were exposed to each of the dispersal concentrations in the Chamber for 4 h at intervals of at least 7 days.

The person in charge of allocation prepared a correspondence table with a table of random numbers that showed the correspondence of each group to the target pollen dispersal levels. Only the person and a technician who managed the control of the pollen count were aware of the target pollen dispersal concentration. The correspondence table, which included the pollen dispersal concentration, was managed by them until the end of the study. The person who managed the control of the pollen grain count started

the pollen dispersal after confirming that all the subjects had entered the Chamber and sat down. The person then dispersed the appropriate number of JC pollen grains on the basis of the correspondence table.

The pollen dispersal concentration in the Chamber was recorded every 3 min during the study period.

Assessment of Efficacy

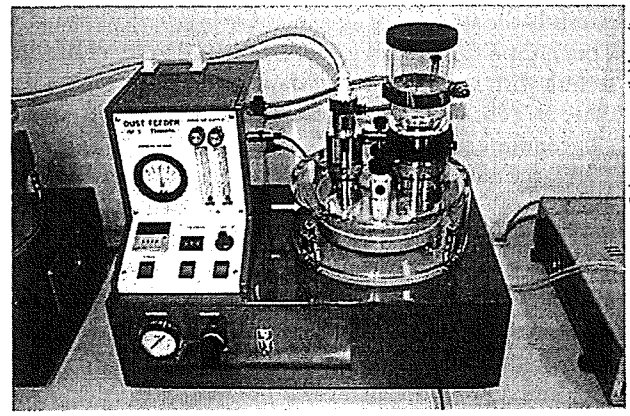
Nasal and ocular symptoms and safety were evaluated in the patients, who were instructed to grade and record the severity of their nasal (sneezing, nasal discharge, nasal obstruction and itchy nose) and ocular symptoms (epiphora and itchy eyes) at regular intervals (immediately before entering the Chamber and at 15-min intervals thereafter) according to the following scale: 0 = none (no symptoms); 1 = mild (symptoms present but easily tolerated); 2 = moderate (awareness of symptoms; bothersome, but tolerable); 3 = severe (definite awareness of symptoms; difficult to tolerate, but does not interfere with the activities of daily living), and 4 = very severe (difficult to tolerate and interferes with the activities of daily living). The mean of the sum of the scores for the four nasal and two ocular symptoms was calculated as the total symptom score (TSS), the mean of the sum of the scores for the nasal symptoms as the total nasal symptom score (TNSS), and the mean of the sum of the scores for the ocular symptoms as the total non-nasal symptom score (TNNSS).

The interval from the time of entry of a patient into the Chamber to the occurrence of the first nasal symptom (any of the 4 nasal symptoms) or the first ocular symptom was designated as 'time to occurrence of symptoms', and the time was recorded for each patient.

The patients were instructed to blow their noses with tissue paper given in advance to each of them. The tissue paper used was recovered in plastic bags at regular intervals (every 30 min). The difference in the weight of the tissue paper measured before and after use was calculated to express the amount of nasal discharge in each subject. Each participant actually counted and recorded the frequency of sneezes at regular intervals him/herself.

Statistical Analysis

The results of the time to the occurrence of symptoms after being exposed to one of the three target dispersal concentrations were compared by the Kruskal-Wallis test in both the summer and winter seasons. In the tests conducted during the summer and winter seasons, the cumulative incidence of the symptoms observed at each pollen dispersal level was analyzed by the Kaplan-Meier method, and the difference in the time to symptom occurrence and the incidence of symptoms at each pollen dispersal level between the summer and winter studies were analyzed by the log-rank test. Significant differences in TSS and TNSS at each point of observation and each concentration level compared to baseline were analyzed by the Wilcoxon signed-rank test. To evaluate the reproducibility of the nasal and ocular symptoms between the summer and winter studies, the results of TSS, TNSS, the total amount of nasal discharge and the sum of the number of sneezes during the summer and winter periods for the same target pollen dispersal concentration were compared by Spearman's rank-order correlation coefficient test. The correlation coefficient and the p value for each result were determined. Differences with p values <0.05 were regarded as significant.



Color version available online

Fig. 1. Pollen supply system: dust feeder and pollen grains on a turntable (→) are shown.

OHIO Chamber

The OHIO Chamber is a chamber measuring 5 × 5 m (25 m^2) with a height of 2.5 m and a capacity of 12 subjects at the maximum, which allows dispersal of JC pollen at constant target pollen dispersal concentrations. 'OHIO' of the OHIO Chamber is short for the facility, and is a combination of initials of the family names of four doctors (Okubo, Hashiguchi, Ishikawa and Okuda), who were involved in the design and development of this facility. The outside air, passed through activated charcoal and HEPA filters, served as conditioned air, with a ventilation frequency of 20 times per hour.

Stable production of pollen as an aerosol with high pollen concentration and homogeneous dispersion of the aerosol at high concentrations in the indoor air are needed to maintain the stability of the pollen concentrations in the Chamber. The following method was adopted for pollen production: Pollen grains on the turntable (rotating at a constant speed) were aspirated with an ejector using compressed air (dust feeder), and an aerosol with high pollen concentrations was thereby supplied (fig. 1). The aerosol was diluted with the outside air and dispersed in the Chamber. Air flow generator systems aimed at circulating the indoor air were set in the four corners of the Chamber, which allowed even distribution of the pollen at the target pollen dispersal concentration in the Chamber [11]. The target pollen dispersal concentrations were determined by means of the laser particle counter KC-20 (Rion, Tokyo, Japan), which allows real-time determination of the pollen concentration on the basis of the scattered light of laser illuminant.

Results

Patient Characteristics

This study included 24 patients (8 males and 16 females; mean age 38.5 ± 9.9 years) who were assigned to one of three groups, each consisting of 8 subjects. Assess-

Table 1. Number of pollen particles dispersed at the time of exposure during the summer and winter studies

Target pollen level	Actual pollen concentration particles/m³	
	summer	winter
0 grains/m³		
Group A	331 ± 126	
Group B	343 ± 91	ND
Group C	147 ± 50	
4,000 grains/m³		
Group A	4,183 ± 348	4,978 ± 1,811[a] (4,374 ± 427)
Group B	4,199 ± 360	4,517 ± 260
Group C	3,435 ± 356	4,107 ± 293
8,000 grains/m³		
Group A	6,808 ± 602	8,039 ± 655
Group B	6,939 ± 697	8,150 ± 572
Group C	7,642 ± 601	7,974 ± 784
12,000 grains/m³		
Group A	10,303 ± 797	11,808 ± 1,002
Group B	10,615 ± 662	12,377 ± 827
Group C	12,494 ± 566	12,201 ± 942

The subjects were divided into three groups (groups A–C) and were exposed to pollen dispersal. The pollen concentrations were counted by a laser particle counter every 3 min. The number of pollen particles counted in each group was expressed as means ± SD.

[a] The number of pollen particles 150 min after the start of exposure to the pollen was 11,166. This finding was attributed to aspiration of a large amount of pollen into the sensor tube when a subject accidentally hit the sensor tube in the Chamber. It was thus clarified that this high concentration was not due to any technical problem in the method used for the pollen dispersal. The figures in parentheses indicate means ± SD calculated after excluding the abnormally large value at 150 min.

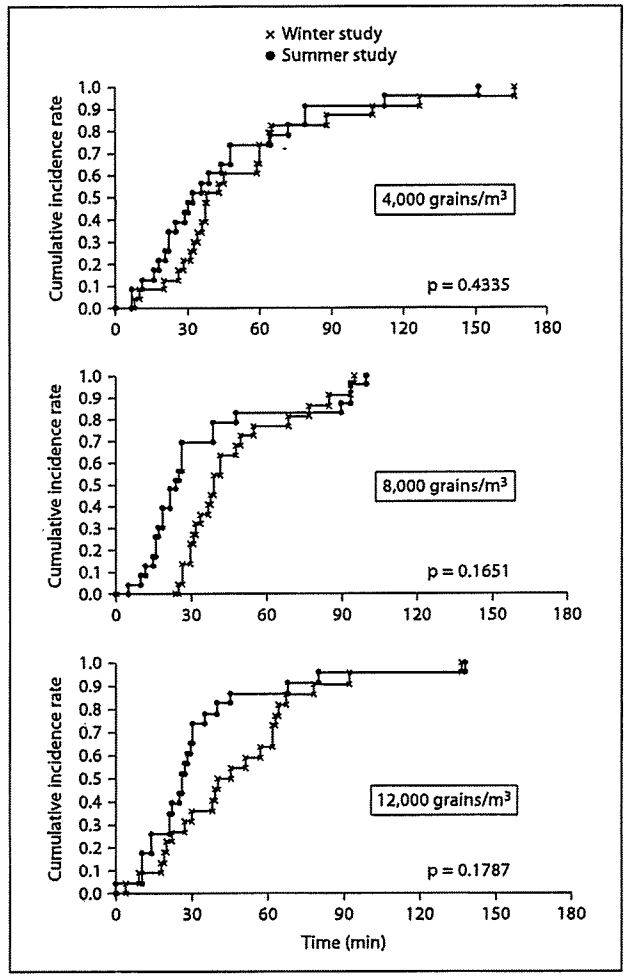


Fig. 2. Comparison of the time to symptom occurrence at the same target pollen dispersal concentration between summer (●) and winter (x) studies using Kaplan-Meier's method. No significant seasonal effect was found at each pollen concentration ($p > 0.05$; log-rank test).

ment of the severity of their symptoms from the records maintained in symptom diaries by individual subjects during the pollen dispersal season revealed that symptoms were mild in 4 subjects, moderate in 8 subjects and severe in 12 subjects. In the summer study, 1 subject could not undergo the study at the target pollen dispersal concentration of 12,000 grains/m³ because of poor physical condition. None of the subjects had any allergic symptoms at the start of the study.

Pollen Dispersal Concentrations, and Temperature and Humidity in the Chamber

Assessment of the target pollen dispersal concentration and the number of pollen grains actually dispersed at the time of exposure during the summer and winter studies revealed that the number of pollen grains actually dispersed was within 10–15% of the target pollen dispersal concentration. In the winter study conducted with the target concentration set at 4,000 grains/m³, an excessively large value of the pollen concentration was recognized 150 min after the start of pollen exposure. This large value was attributed to the aspiration of a massive amount of pollen into the sensor tube in the Chamber when a subject accidentally hit the sensor tube. Thus, it was clarified that the abnormal value was not attributable to any technical failure in the method of pollen dispersal in the Chamber (table 1).

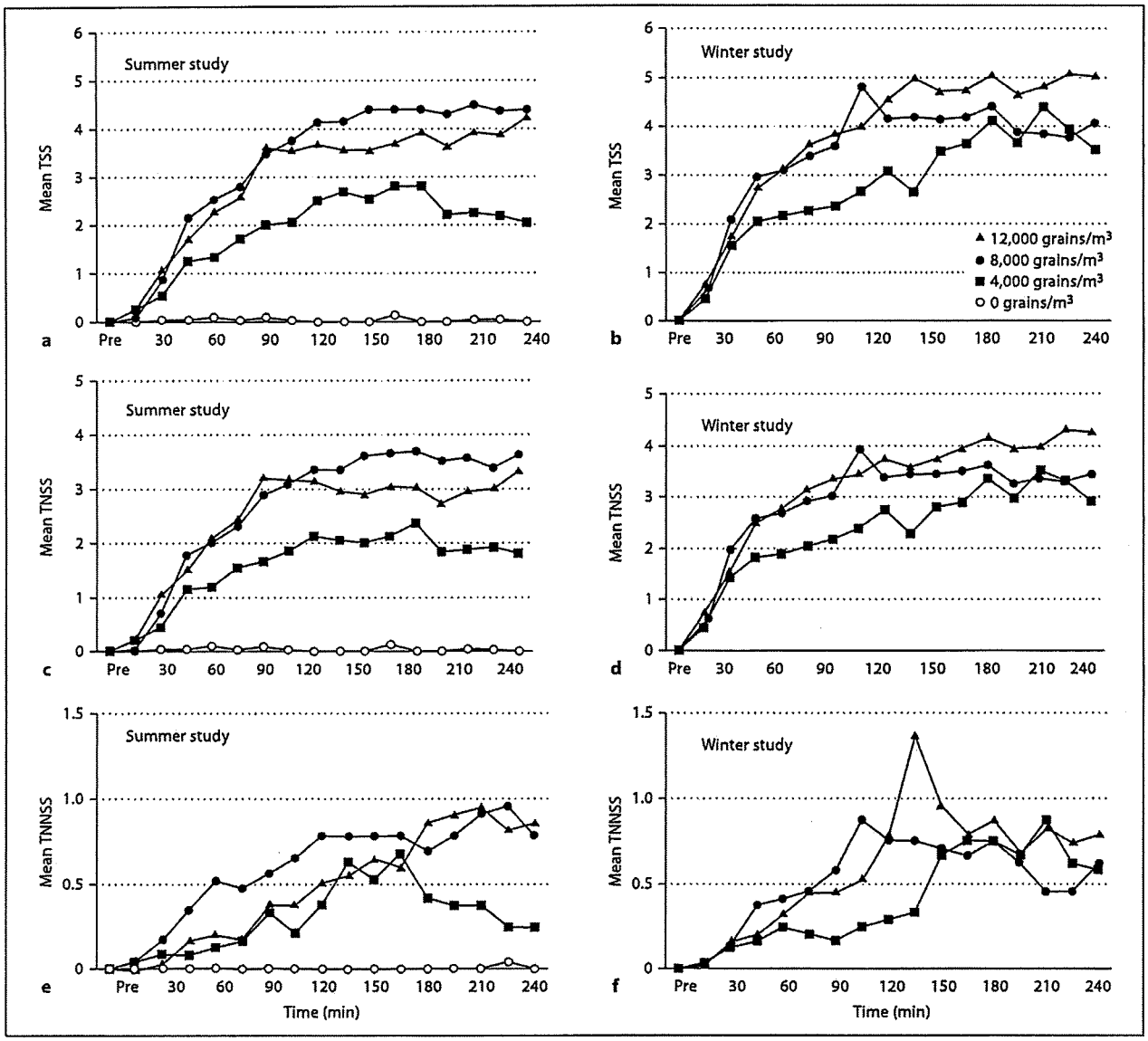


Fig. 3. Mean total symptom scores (TSS) at different pollen concentrations in the summer (**a**) and winter studies (**b**) are shown. None of the subjects developed any symptoms at the pollen concentration of 0 grains/m³ (open circle). TSS increased in a dose-dependent manner for all pollen dispersal concentrations. TNSS and TNNSS at the three different pollen dispersal concentrations in the summer (**c**, **e**) and winter studies (**d**, **f**) are shown. There were no significant differences in the TSS and TNSS between each of the three pollen exposure concentrations both in summer and winter studies. At each concentration, TSS and TNSS were significantly increased compared to baseline from 30 min after exposure.

The mean temperature and humidity values in the Chamber determined at 3-minute intervals in each study were adopted as the values for the analysis. Room temperature was 22.1 ± 0.1°C and relative humidity was 44.4 ± 1.0%, being close within the range of target values.

Time to Symptom Occurrence

With regard to the time to symptom occurrence at each pollen dispersal concentration in the Chamber (table 2), none of the subjects developed any symptoms at the target pollen dispersal concentration of 0 grains/m³.

Table 2. Time to the occurrence of first symptoms at each pollen exposure level

Exposure level	Summer study, min	Winter study, min
0 grains/m^3	NA	ND
4,000 grains/m^3	53.1 ± 37.9	44.0 ± 35.4
8,000 grains/m^3	40.9 ± 22.7	35.0 ± 25.3
12,000 grains/m^3	59.0 ± 53.4	34.1 ± 28.9

Data are given as means ± SD. NA = Not available; ND = not done.

Table 3. Correlation coefficients between the summer and the winter studies for the TSS, TNSS, the amount of nasal discharge and the frequency of sneezing

Pollen concentration	Correlation coefficient[a]	p value
TSS		
4,000 grains/m^3	0.659	<0.001
8,000 grains/m^3	0.678	<0.001
12,000 grains/m^3	0.757	<0.001
TNSS		
4,000 grains/m^3	0.631	<0.001
8,000 grains/m^3	0.685	<0.001
12,000 grains/m^3	0.749	<0.001
Amount of nasal discharge		
4,000 grains/m^3	0.538	<0.001
8,000 grains/m^3	0.408	<0.001
12,000 grains/m^3	0.589	<0.001
Frequency of sneezes		
4,000 grains/m^3	0.062	0.226
8,000 grains/m^3	0.261	<0.001
12,000 grains/m^3	0.112	0.032

There are correlations in the TSS, TNSS and the amount of nasal discharge, suggesting favorable reproducibility of symptoms at each pollen concentration.

[a] Spearman's rank-order correlation coefficient test.

There were no significant differences in the time to the occurrence of symptoms among the three other target pollen dispersal concentrations examined (4,000, 8,000 and 12,000 grains/m^3) either in the summer or the winter study ($p = 0.297$ and 0.390, respectively; Kruskal-Wallis test). For the same pollen dispersal concentration, there was also no significant difference in the time to symptom occurrence between the summer and the winter study (Kaplan-Meier estimates and log-rank test; fig. 2).

Symptoms Observed at the Various Pollen Dispersal Concentrations and Reproducibility of the Symptoms

At the target pollen dispersal concentration of 0 grains/m^3 (placebo), none of the participants developed any nasal or ocular symptoms. Therefore, no attempt was made to study this target pollen dispersal concentration in the winter study.

The severity of symptoms increased in the subjects as the target pollen dispersal concentration increased. The tendency during the winter study was similar to that during the summer study; at all the target pollen dispersal concentrations examined, the symptom scores began to increase almost immediately from 30 min after the start of exposure. The TSS reached a plateau 90–120 min after the start of the study (fig. 3a, b).

The time course of changes in the TNSS showed the same tendency as that of the TSS in both the summer and the winter studies (fig 3c, d). Changes in the TNNSS were slower compared to those of the nasal symptoms, and in most cases reached a plateau approximately 120 min after the start of pollen dispersal in the patients exposed to target concentrations of 8,000 and 12,000 grains/m^3, and about 150–165 min after the start of pollen dispersal in the patients exposed to the target concentration of 4,000 grains/m^3 (fig. 3e, f). We analyzed whether or not the symptom scores (TSS and TNSS) increased significantly compared with the baseline scores for each pollen dispersal concentration, and in the summer and winter studies, significant increases in the symptom scores (TSS and TNSS) were observed for all the pollen exposure concentrations examined from 30 min after the start of exposure (Wilcoxon signed-rank test: $p < 0.05$).

The total amount of nasal discharge determined in each subject every 60 min after the start of the pollen exposure increased in a time-dependent manner, reaching a plateau approximately 120 min after the patients had entered the Chamber (fig. 4a). The frequency of sneezing, which was measured at ~30-min intervals after the start of exposure, started to increase from 30 min after the start of exposure but remained mostly unchanged after 60 min (fig. 4b).

The correlation coefficients between the summer and the winter studies were high for the TSS, TNSS and the amount of nasal discharge at each pollen dispersal concentration examined. There were no significant differences in the TSS and TNSS between the summer and the winter studies at the pollen dispersal concentrations examined (table 3). The results suggested favorable reproducibility of symptoms at each of the pollen dispersal

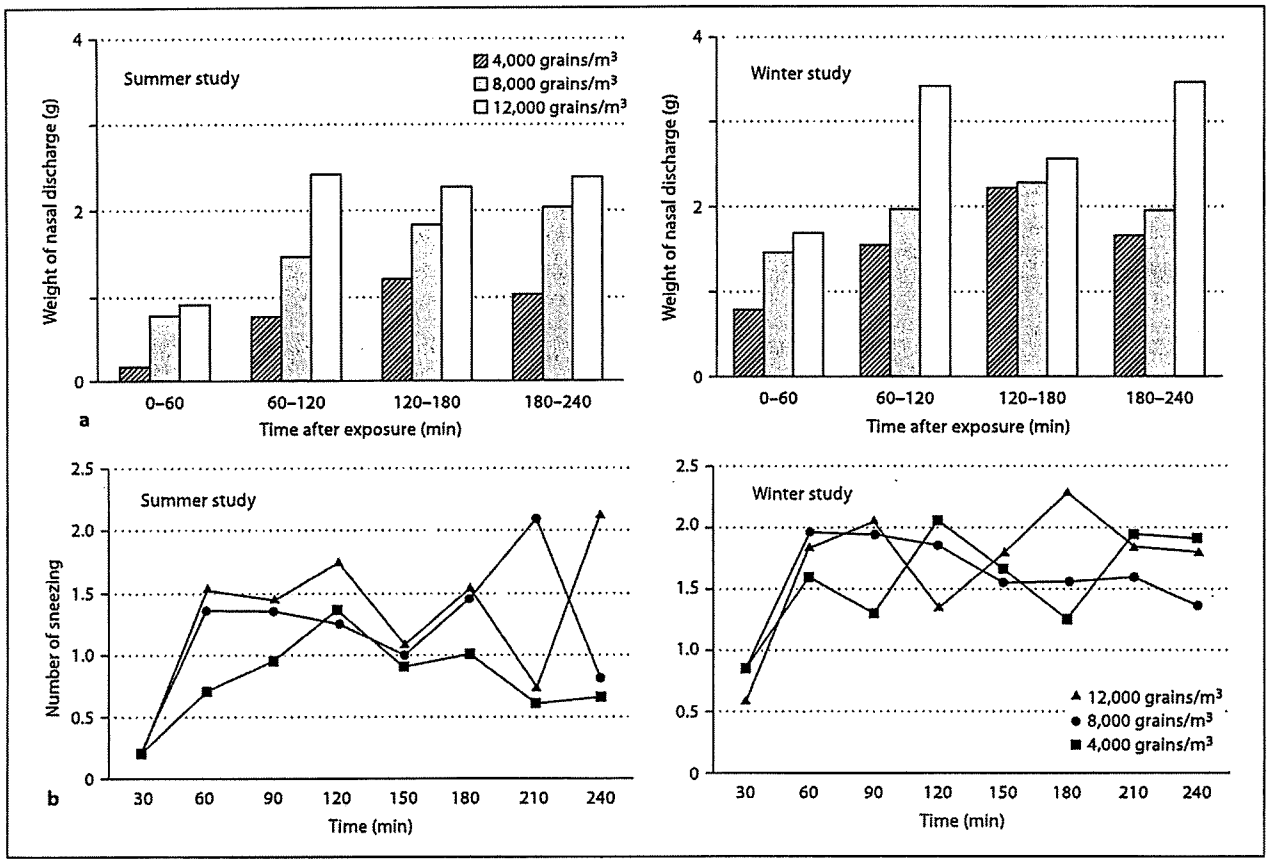


Fig. 4. a The amount of nasal discharge measured every 60 min at three different pollen dispersal concentrations in the summer and winter studies. For the same pollen dispersal concentration, the amount of nasal discharge did not significantly differ between the summer and winter studies, indicating that there were correlations in the results (Spearman's rank-order correlation coefficient test). **b** The frequency of sneezing recorded every 30 min at three different pollen dispersal concentrations did not significantly differ between the summer and winter studies.

concentration examined, whereas there was no correlation in the frequency of sneezing between the summer and winter studies.

Discussion

This study was designed to identify the validity of tests conducted in patients exposed to JC pollen in the OHIO Chamber. The results of the exposure tests conducted twice during non-pollen seasons in patients with JC pollinosis demonstrated that symptoms characteristic of pollinosis manifested consistently and that the severity of the symptoms for a given JC pollen grain dispersal concentration were reproducible.

The number of JC pollen grains dispersed annually has been reported to be in the range of 500–2,000 grains/m^3 (measured by a different type of laser counter, KH-3000, Ministry of the Environment, Japan) in the suburban districts of Tokyo, in which the number of JC pollen grains dispersed is usually large. These numbers are equivalent to the range of 3,000–12,000 determined by the KC-20 leaser counter used in the OHIO Chamber system [11]. Based on these findings, the pollen dispersal concentrations used in this study (maximum of 12,000 grains/m^3) are considered to be valid and not too high. Therefore, we selected four different pollen dispersal concentrations, namely 0, 4,000, 8,000 and 12,000 grains/m^3, aiming to assess the validity of the pollen dispersal concentrations. With regard to the duration of exposure,

the results of the validation study reported by Krug et al. [9] were used as reference values. They conducted a 4-hour exposure test and showed that the manifestation of the symptoms in their subjects reached a plateau after approximately 2 h of exposure. Based on these results, observation of the symptoms for at least 2 h was considered necessary, and a test exposure time of 4 h was adopted.

The pollen dispersal concentration in the OHIO Chamber was measured using a laser particle counter (KC-20), allowing real-time determination of the pollen dispersal concentration. The results of the pollen dispersal concentrations are transmitted instantaneously to the operator in the operating room outside the Chamber, allowing a constant pollen concentration to be maintained in the Chamber. In the present test, the difference between the actual numbers of pollen grains dispersed and the target pollen dispersal concentrations in the Chamber was within ~10% during both the summer and winter periods, which is acceptable. At the target pollen dispersal concentration of zero, however, 150–350 particles were detected in the Chamber, which were approximately 10 μm in size. Taking into consideration the approximately 30-μm size of JC pollen grains, these particles detected in the Chamber were not considered to be JC pollen, but rather particles brought into the Chamber when the subjects entered the Chamber. Krug et al. [9] showed similar results in their validation study, in which they used not only a laser particle counter, but also a system of two rotating rod samplers to count pollen numbers light-microscopically.

Chamber temperature and humidity could be kept constant within a range of ±0.1°C and ±1.0%, respectively, in both the summer and winter, being also satisfactory.

The volunteers were exposed to three distinct pollen dispersal concentrations during the summer and winter periods aiming to assess the reproducibility of the symptoms developing in response to pollen exposure in the OHIO Chamber. One patient with mild symptoms of JC pollinosis showed marked reaction only to the pollen dispersal concentration of 4,000 grains/m^3 in the winter study. This patient showed no or few symptoms at the higher pollen dispersal concentrations. One possible reason for this is that the low temperature of the outside air increased this patient's nasal hypersensitivity [15]. The other patients showed reproducible symptoms during the summer and winter studies.

The results of the present study indicate the reproducibility of the symptoms at all the pollen dispersal concentrations examined. Krug et al. [9] exposed the subjects to the same pollen dispersal concentration 5 times to observe the reproducibility of the symptoms. We conducted validation tests in different seasons in patients who were not informed of the pollen dispersal concentrations in order to avoid the influence of their experiences and recollections on the self-assessment.

Our assessment methods included TSS evaluation, which is a subjective method, as well as the determination of the amount of nasal discharge and the frequency of sneezing, which serve as objective indicators. Recent studies conducted in the exposure chambers in Austria [16] and Germany [17] have also mainly used TNSS to evaluate patient symptoms before and after treatment with antihistamines. Our TSS and TNSS levels were lower than those in reports on the efficacy of antihistamines for allergic rhinitis. One of the reasons for this finding could be that subjects with varying degrees of symptom severity were enrolled in our study, since the aim of this study was not to determine the efficacy of antiallergic drugs. In other words, there were 4 patients with very mild symptoms following pollen exposure in the natural environment. TNSS was as low as 1 even after exposure to extremely high pollen concentrations in these 4 patients. On the other hand, while examining the efficacy of antiallergic drugs using the OHIO Chamber in the future, it would be necessary to select the subjects who will show an increase in TNSS with increasing pollen exposure.

A correlation between allergic rhinitis and asthma has been established previously [18]. Due to their diameter (~ 30 μm), JC pollen grains are retained in the nasal cavity and not considered to invade the lower airways. Therefore, the development of asthma as a result of JC pollen inhalation is quite improbable. In the past 2 decades, while we have experienced many years with massive dispersal of JC pollen, there have been no or few reports on the occurrence of asthma during these massive JC pollen dispersals. Krug et al. [9] and Horak et al. [19] have demonstrated that there were no changes in the respiratory function of subjects in studies using pollen exposure chambers. In this study, none of the volunteers in the OHIO Chamber developed lower airway symptoms during the pollen exposure according to their subjective and objective symptoms.

In conclusion, we have shown the reproducibility of the severity of symptoms in the same patients in the OHIO Chamber during the summer and winter studies, both of which are non-JC pollen dispersal seasons. Reproducibility was recognized regardless of the severity of the symptoms in the subjects. Relatively stable symptoms occurred at concentrations of at least 4,000 grains/m^3 in the present study. In particular, exposure to 8,000 grains/

m^3 yielded relatively high TNSS. The symptoms reached a plateau approximately 90–120 min after the start of exposure, indicating that the optimum amount and duration of exposure for testing in the OHIO chamber are at least 8,000 grains/m^3 and 120 min, respectively. The results also demonstrated the reactivity and safety of several JC pollen exposure levels in the OHIO chamber.

This study also allowed the assessment of the efficacy of antiallergic drugs for JC pollinosis patients and the effects of medications administered before the JC pollen dispersal season suppressing the manifestation of symptoms. The present study indicates that the exposure chamber has the potential to contribute significantly to basic and clinical studies of JC pollinosis.

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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検討する目的に、舌下免疫療法の詳細な検討と制御性T細胞による免疫学的反応を検討した。舌下免疫療法はスギ花粉飛散総数にかかわらず少ない薬物使用で症状を軽減し、経年治療による効果が認められた。また、治療は3年で終了せず4年目も行う方がよかった。小児例(n=61)では32%が無投薬で、約80%で有効で、小児で非常に有効であった。舌下免疫例では、誘導性制御性T細胞であるIL-10産生性のTr1が増加し、Proliferation assayからIL-10がfunctionalであると示された。

A. 研究目的

我々は、舌下免疫療法の詳細な位置づけを検討する目的に、花粉飛散数の影響、継年治療の意義、治療期間について解析した。また、本邦初の試みとしての小児スギ花粉症に対する舌下免疫療法の対症例数を増やし、小児に対する安全性と効果を検討した。さらに、機序解明の目的に誘導性制御性T細胞に注目し、IL-10の関与を検討した。

B. 方法

I) 成人舌下免疫療法での検討　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、中等度飛散年(2008年 2,691個/cm2)と大量飛散年(2009年 11,941個/ cm2)で毎日の症状スコアから花粉飛散数の影響を検討した。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。

II) 小児スギ花粉症に対する舌下免疫療法　2008年までの15例に加えて、新たに46例をエントリーし、合計61例で検討した。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。

III) 舌下免疫療法機序の基礎的研究　舌下免疫療法患者でスギ花粉飛散期に $CD4^+$細胞を分離し、FACSにより誘導性制御性T細胞でIL-10を多く産生する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。

C. 結果

I) 成人舌下免疫療法での検討

1) 花粉飛散数の影響　毎日の症状スコアでは中等度飛散年には皮下免疫療法の効果が最も高く、次いで舌下免疫療法、初期療法であった。大量飛散年には3群の差が縮まったが、傾向は同じであった。一方で、薬物スコアは舌下免疫療法と皮下免疫療法ともに低かった。この結果から飛散総数にかかわらず、舌下免疫療法は薬物使用を少なくし症状を軽減できると考えられた。

2) 継年治療の意義　2009年に治療開始して1年目、2年目、4年目となる3群の比較で、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た。症状薬物スコアは3群間で有意な差が認められ、経年治療による効果が確認された。

3) 治療期間　症状スコアと薬物スコアは全般に4年目群で良好であった。4年目群の例数が少なく症状スコアと薬物スコアに有意差がでなかったが、症状薬物スコアは4年目群が3年終了群より有意に良好であった。治療は3年で終了せず4年目も行う方がよいと考えられるが、大量飛散年での結果であり、更なる検討が必要である。

II) 小児スギ花粉症に対する舌下免疫療法

全例が副作用なく安全に自宅で治療できた。2009年は大量飛散年であったため2008年の結果ほど有効ではなかったが、症状日記から算出する平均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点であり、大量飛散年としては良好な成績であった。また、薬物スコアは非常に低く、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。アンケートによる効果判定では、全体の80%で良好であり、1年目例でも効果が認められた。

III) 舌下免疫療法機序の基礎的研究

$CD4^+$T細胞に対するTr1の割合は、健常人より未治療花粉症で少なく、舌下免疫療法では健常人と同等に多くなっていた。舌下免疫療法により

IL-10 産生性の Tr1 が増加すると考えられた。また、Crj1 の刺激だけでなく、CD3/CD28 刺激においても Tr1 比率は大きく、抗原非特異的な刺激にも反応することが確認された。この結果は、スギ花粉免疫療法患者では、その他のアレルゲン刺激が加わった際にも免疫寛容に向かえる可能性を示している。Proliferation assay では舌下免疫療法は、健常人と同等で未治療花粉症より弱い細胞分化が認められたが、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。

D. 考察

舌下免疫療法には課題も多いが、本研究を通じて解決できたものも多い。他治療との比較から、皮下免疫療法には効果で及ばないが、初期療法よりは良く、何よりも薬物使用量を少なくできることは患者の負担を小さくできる。小児により効果的であれば、その利得も大きいと考えられる。また、始めて誘導性制御性 T 細胞が増えていることが判明し、IL-10 を介した免疫寛容があると推測できた。

E. 結論

本研究で舌下免疫療法の位置づけがよりはっきりし、治療の方向性が明確となった。また、小児例は成人例よりも効果が高いことが示された。基礎的研究により舌下免疫療法の作用機序に Tr1 を介した IL-10 の作用が示唆された。

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

Letter to the Editor

Induction of IL-10–producing regulatory T cells with TCR diversity by epitope-specific immunotherapy in pollinosis

To the Editor:

Specific peptide-based allergen immunotherapy is currently being used for several allergic diseases. Immunotherapy for Japanese cedar pollinosis was undertaken using sublingual application of a pool of pollen peptides containing 7 T-cell epitopes from Cry j 1 and Cry j 2, which are the major Japanese cedar pollen allergens, and its clinical efficacy on seasonal allergic rhinitis was evaluated.[1] Interestingly, some patients undergoing immunotherapy showed reduced sensitivity to other allergens in addition to Japanese cedar pollen. Allergen-specific regulatory T cells have been suggested to play a role in this immunoprotection,[2-5] but the precise mechanism is not fully understood.

IL-10–producing regulatory T cell (Tr1) is one type of acquired regulatory T cells with unclear characteristic phenotypes. In the current study, the Tr1 population percentage was compared

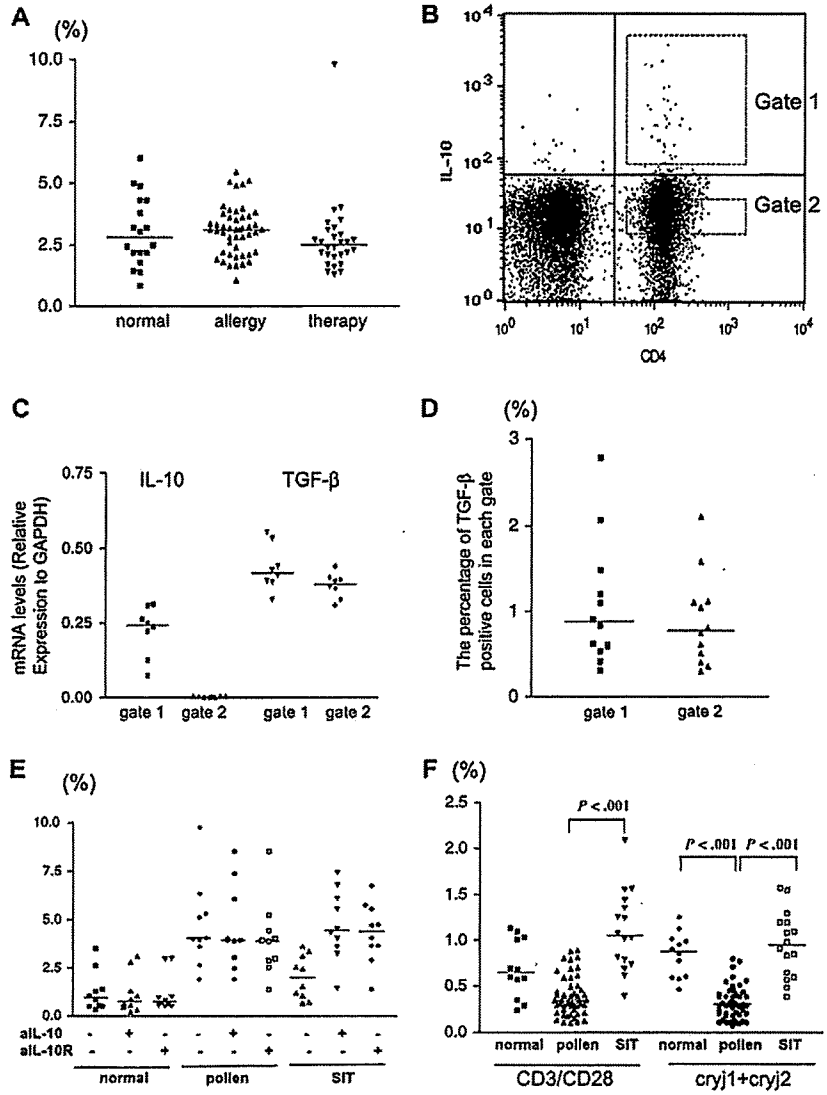

FIG 1. A, The percentage of forkhead box $P3^+CD25^{high}CD4^+$T-cells (naturally occurring regulatory T cells) was unchanged in patients with pollinosis and in those undergoing SIT compared with controls. B, Tr1 *(gate 1)* and non-Tr1 *(gate 2)* cells are gated. C, Tr1 cells show equivalent expression of TGF-β mRNA compared to non-Tr1 cells, different from that of IL-10. D, When cells were costained with anti–TGF-β antibody, the percentage of TGF-β–positive cells was similar between Tr1 *(gate 1)* and non-Tr1 *(gate 2)*. E, $CD4^+$ lymphocyte proliferation was significantly suppressed in PBMCs from patients who received SIT compared with patients with pollinosis. This suppressive function was abolished by the addition of anti–IL-10 neutralizing antibody or anti–IL-10 receptor antibody. F, The percentage of Tr1 cells was significantly decreased in patients with untreated pollinosis compared with normal controls and increased in patients receiving SIT.

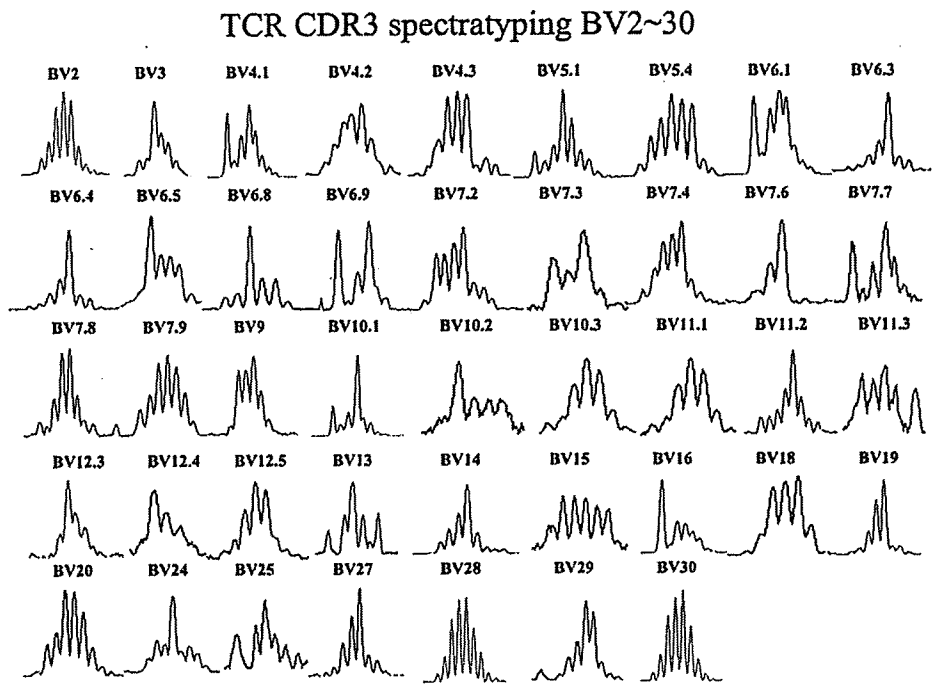

FIG 2. T-cell receptor complementarity-determining region 3 *(CDR3)* size spectratyping analysis of a Tr1 population from patients undergoing immunotherapy. A Gaussian distribution of CDR3 lengths was observed in many BV subfamilies, except for oligoclonal expansion in some BV subfamilies, indicating preservation of a highly diverse T-cell receptor repertoire.

among untreated patients with pollen allergy, patients undergoing immunotherapy, and healthy subjects. The results showed that the number of Tr1 cells was significantly increased in patients receiving immunotherapy compared with the other groups. In addition, the diversity of the T-cell receptor repertoire in the Tr1 population from patients undergoing immunotherapy was investigated.

Seventy-five patients from the Departments of Dermatology and Otorhinolaryngology of Mie University Hospital were enrolled (15 patients with pollen allergy receiving sublingual immunotherapy [SIT] as described, and 60 sensitive to Japanese cedar). Thirteen healthy controls were also recruited (see details in this article's Table E1 in the Online Repository at www.jacionline.org). Diagnosis was based on clinical symptoms and serological results. Blood was sampled after obtaining written informed consent from all subjects, and the investigational protocol was approved by the Institutional Review Board of Mie University Hospital. Healthy volunteers had no history or subjective symptoms of atopic dermatitis or pollen allergy.

Recent studies have shown a significant decrease of forkhead box $P3^+CD25^{high}CD4^+$T cells, naturally occurring regulatory T cells in symptomatic patients with atopic dermatitis or bronchial asthma.[6] However, the percentage of naturally occurring regulatory T cells (forkhead box $P3^+CD25^{high}CD4^+$T cells/$CD4^+$T cells) remained unchanged in patients with pollinosis and in those receiving SIT compared with controls (Fig 1, *A*). In the current study, the IL-10–producing Tr1 cell population was characterized by flow cytometry and cell sorting. PBMCs from patients and normal donors were isolated from heparinized venous blood by density gradient centrifugation using Ficoll (for antibodies and reagents, see Methods in this article's Online Repository). PBMCs were cultured in RPMI 1640 medium with L-glutamine supplemented with 100 U/mL penicillin, 100 U/mL streptomycin, and 10% human type AB serum. These cells were stimulated with 100 ng/mL soluble anti-CD3 Ab plus 100 ng/mL anti-CD28 Ab, or 50IU Cry j 1 and Cry j 2, for 8hours. After culture, cells were collected and incubated with anti–CD4–fluorescein isothiocyanate and phycoerythrin-conjugated IL-10 secretion antibody. Immunophenotypic analysis was performed by using a Becton Dickinson FACScan instrument and cell sorting by using a FACS Aria cell sorter (Becton Dickinson, Mansfield, Mass). The Tr1 population was gated (gate 1) as shown in Fig 1, *B*. When PBMCs were stimulated with CD3/CD28 antibodies, these 2 sorted populations showed similar expression of TGF-β compared with non-Tr1 cells (gate 2) on the basis of quantitative PCR or intracellular staining with anti–TGF-β antibody in normal controls (Fig 1, *C and D*). Subsequently, cellular proliferation was evaluated. PBMCs (10^6/mL) were first labeled with 10 μmol/L Carboxyfluorescein Succinimidyl Ester and stimulated with Cry j 1 and Cry j 2 as discussed. After 4 days of culture, PBMCs were stained with Peridinin-chlorophyll-protein complex-conjugated anti-CD4 antibody, and the percentage of $CD4^+$ lymphocyte proliferation was measured. The proliferation was significantly suppressed in patients receiving SIT compared with patients with pollen allergy (Fig 1, *E*). This suppressive effect was neutralized by the addition of 2μg/mL anti–IL-10 antibody or anti–IL-10 receptor antibody, confirming that the suppression of reactivity against cedar pollen in patients receiving SIT is IL-10–dependent (for the proliferation assay, see Methods in this article's Online Repository). The percentage of circulating Tr1 cells ($IL\text{-}10^+CD4^+$T cells/$CD4^+$T cells) was significantly decreased in untreated patients with allergy compared with

normal controls, and the level was increased in patients receiving SIT compared with untreated patients with allergy (Fig 1, *F*). This elevation of Tr1 cells in SIT-treated patients was observed when PBMCs were stimulated not only with Cry j 1 and Cry j 2 but also with CD3/CD28 antibodies, and this may be 1 reason why some patients undergoing immunotherapy for Japanese cedar pollen showed reduced sensitivity to other allergens. We also performed phenotypic characterization of the identified Tr1 cells by flow cytometry; interestingly, Tr1 cells showed more skin and gut–homing tendency than the non-Tr1 population (see this article's Fig E1 on the Online Repository at www.jacionline.org).

We next performed complementarity-determining region 3 size spectratyping analysis by using established β-variable primers to investigate the diversity of the T-cell receptor repertoire in the Tr1 populations expanded or changed by immunotherapy.[7,8] Total RNA was extracted from the sorted Tr1 population, and cDNA was prepared as previously reported.[7,8] Fig 2 shows a representative spectratype from Tr1 cells taken from SIT-treated patients. Although the Tr1 population percentage was increased, a Gaussian distribution of complementarity-determining region 3 lengths could be observed in many BV subfamilies examined, except for oligoclonal expansion (2-4 peaks in spectratyping) in some BV subfamilies, indicating preservation of a highly diverse T-cell receptor repertoire (see this article's Fig E2 in the Online Repository at www.jacionline.org for spectratypes from other population).

Tr1 cells can be generated in the presence of locally produced IL-10 released from Tr1 cells.[4] Tr1 cells block cellular proliferation not via cell-to-cell contact but via cytokine-mediated mechanisms (eg, IL-10). The results of the current study may explain the mechanism of suppression against multiple allergens induced by epitope-specific immunotherapy in patients with pollinosis. Specific immunotherapy blocks the allergen specific IgE and induces an allergen-specific IgG response, which is induced by IL-10 from Tr1 cells.[2,3]

In conclusion, IL-10–producing Tr1 cells induced by specific immunotherapy play multifunctional roles in suppressing allergic reactions. We have shown here that patients undergoing specific immunotherapy have increased IL-10–producing Tr1 cell populations with high T-cell repertoire diversity, suggesting that this therapy induces tolerance to multiple allergens.

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METHODS

Antibodies and reagents

Ficoll was purchased from Sigma (St Louis, Mo), and the IL-10 secretion assay kit was from Miltenyi Biotec (Auburn, Calif). Cell surface staining buffer contains 0.1 mol/L PBS and 2% FCS (Biowest, Nuaillé, France). mAbs to CD4–fluorescein isothiocyanate, CD25-phycoerythrin, and forkhead box P3-phycoerythrin-Cy5 were purchased from eBioscience (San Diego, Calif). Antibody against TGF-β–Peridinin-chlorophyll-protein complex was purchased from R&D (Minneapolis, Minn). The cells were stimulated with 100 ng/mL soluble anti-CD3 plus 100 ng/mL soluble anti-CD28 (BD Biosciences, San Jose, Calif), or 50 IU Cry j 1 and Cry j 2 (Torii, Tokyo, Japan). Cells were cultured in a final volume of 200 μL RPMI 1640 medium with L-glutamine supplemented with 5 mmol/L HEPES, 100 U/mL penicillin, 100 U/mL streptomycin (Invitrogen, Carlsbad, Calif), and 10% FCS.

Real-time quantitative PCR

RNA was isolated by using ISOGEN (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. Total RNA 1 μg (A260/A280 = 1.7-2.0) was reverse-transcribed with oligo-dT primers and the SuperScript III First-Strand Synthesis System (Invitrogen) in a final volume of 20 μL. Quantitative real-time PCR was performed with a TaqMan by using ABI gene expression assays (Applied Biosystems, Foster City, Calif) according to the manufacturer's instructions. GAPDH was used as a control for cDNA input.

Proliferation assay

PBMCs (10^6/mL) were initially labeled with 10 μmol/L CFSE and then stimulated with 50 IU Cry j 1 and Cry j 2 with or without 2 μg/mL anti–IL-10 neutralizing antibody or anti–IL-10 receptor antibody (R&D) for 4 days. PBMCs were collected and stained with CD4-PerCP antibody (BioLegend, San Diego, Calif). Cell proliferation was analyzed by a CellQuest flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The percentage of proliferative cells was calculated by dividing $CD4^+$ proliferative lymphocytes by the total number of $CD4^+$ lymphocytes (n = 10 per each population).

Statistical analysis

Statistical analysis was performed by using the Kruskal-Wallis nonparametric ANOVA test with *post hoc* analysis with the Dunn multiple comparison test. A *P* value less than .05 was considered statistically significant.

Complementarity-determining region 3 spectratyping analysis

T-cell receptor BV segments were amplified with 1 of 43 BV subfamily-specific primers and constant-variable primer recognizing both CB1 and CB2 regions. The sequences of BV primers and fluorescent CB primer have been previously described.[E1,E2] PCR products were applied to a 5% polyacrylamide sequencing gel, and the size distribution of each fluorescent PCR product was determined by electrophoresis on an automated 377 DNA sequencer (Applied Biosystems). In this technique, an amplified TCR BV subfamily migrates as a series of bands, each one corresponding to a different complementarity-determining region 3 length separated from one another by 3 nucleotides. Data were analyzed by using the GeneScan software (Applied Biosystems), which assigns a size and peak area to the different PCR products.

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