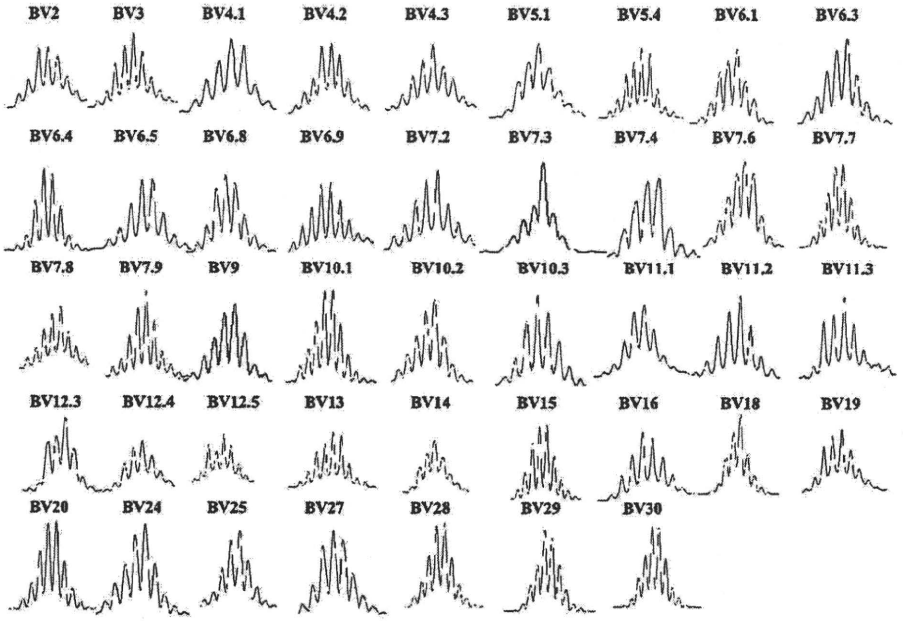

FIG E2. *(Continued)*

TABLE E1. Patients' information

	Normal	Allergy	SIT
N	13	60	15
Age (y)	38.4 ± 6.1	26.8 ± 5.7	39.1 ± 14.5
Male and female	7 and 6	43 and 17	5 and 10
TNSS	0	4.34 ± 1.61	2.21 ± 1.85

Nasal symptoms of sneezing, nasal discharge, and nasal congestion were recorded every day. Symptoms were categorized on a 5-point scale (0, none; 1, mild; 2, moderate; 3, severe; 4, extremely severe) according to practical guidelines for the management of allergic rhinitis in Japan. The average of total nasal symptom score *(TNSS)* for the 3 nasal symptoms in the cedar pollen season of February and March 2008 was calculated.[E3]

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(総合)研究報告書

「リアルタイムモニター花粉数の情報のあり方の研究と舌下ペプチド・アジュバント療法の臨床研究」
舌下免疫療法を再開すると治療効果は継続・増強するか

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研究要旨
舌下免疫療法は有効性・安全性の面で優れた治療方法であることがこれまで臨床研究で確認できた。今後はどのぐらい継続すればよいのか、季節外にも投与が必要かなど、実際の診療に沿った投与スケジュールを試みていく必要がある。
今回我々は舌下免疫療法を一度中断した症例に再度投与を行い、良好な成績を得ることができたので報告する。

A. 研究目的

花粉症を治癒に導くと考えられている方法は唯一免疫療法だけである(IT: Immunotherapy)。しかし現行の IT が一般的な治療にならない理由は、ショックを起こす危険性があることであり、このような IT の副作用を減少させるために欧米では代替免疫療法が試みられている。特に舌下免疫療法(SLIT, Sublingual Immunotherapy) は二重盲検比較試験で有効性が証明されている。SLIT で大量の抗原を口腔粘膜から吸収させると、治療効果があり、副作用の危険性も極めて少ない。
従来の注射法による免疫療法でも一度治療を中断した後に改めて治療を再開する症例がある。その場合、増量期を短くでき、効果発現が速やかになる。舌下免疫療法を再開した場合

B. 方法

舌下免疫療法 (SLIT) についてインフォームドコンセントを得たスギ花粉症ボランティアを対象にプラセボ対照二重盲検比較試験を行った。治療に使用した抗原エキスは、鳥居薬品製標準化スギ花粉治療用エキスを使用した。
舌下免疫療法を再開した症例は、増量期を設けず治療開始濃度は 2000JAU である(追加舌下免疫)。一方、初回治療群では (二重盲検比較試験　プラセボ群および実薬群)、治療開始濃度は 2JAU/ml とし 1 週ごとに 10 倍高濃度のエキスを投与し 2000JAU/ml まで漸増し、花粉飛散季節では最終的に 1 週間に 1 回の投与とした。
二重盲検比較試験でのプラセボ 14 人、二重盲検比較試験実薬群 36 人、追加治療群 71 人だった。季節中の症状について症状スコア、重症度スコアによって評価した。試験デザインについては、日本医科大学倫理委員会の承認を得て実施した。

C. 結果

2 月初めから 3 月上旬まではプラセボ群、実薬群、追加舌下免疫群共に総鼻症状スコア(TSS)に明らかな差を認めなかった。しかし、スギ花粉飛散が多くなる 3 月中旬以降には、プラセボがもっとも症状が強く、次に実薬群、最も軽症だったのは追加舌下免疫群だった。この傾向は 4 月下旬まで継続した (図 1)。経過中、局所および全身性の副作用は発生しなかった。

D. 考察 :

IT は唯一の根治的治療法であるが、アナフィラキシーショックを起こす危険性があるので一般医家には普及していない。今回の結果から SLIT は季節中の症状の重症化を防ぐことによって、花粉飛散期の症状を軽症化している傾向があった。花粉症患者はスギ花粉が多く飛散する時期でも QOL を悪化させることが少なく、シーズンを過ごすことが可能になると考えられる。また副作用もなく安全に治療できる。
舌下免疫療法を再開すると、治療効果が速やかに現れる。したがって、従来の注射法のように季節以外にも維持量を定期的に注射しなくても、季節前に舌下免疫療法をリスタートすれば即効性のある効果が期待できる。

E. 結論

スギ花粉症の症状を抑制し，薬物の使用量を減少させられる SLIT は医療経済上でも有用な方法である。今後は SLIT の長期的な効果を評価し、多くの症例を長期的に検討することによって作用メカニズムの解明や治療スケジュールの確立をしなければならない。

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G. 知的財産権の出願・登録状況

1. 特許取得
なし

2. 実用新案登録
なし

3. その他
なし

Biosci. Biotechnol. Biochem., 73 (9), 1971–1977, 2009

Efficacy of Oral Administration of a Heat-Killed *Lactobacillus gasseri* OLL2809 on Patients of Japanese Cedar Pollinosis with High Japanese-Cedar Pollen-Specific IgE

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A randomized, double-blind, placebo-controlled clinical trial was conducted to determine whether oral administration of heat-killed *Lactobacillus gasseri* OLL2809 would affect the immune response and reduce the symptoms of Japanese cedar pollinosis (JCP) in subjects with JCP. Following a 1-week pre-observation period, the subjects were randomly divided into two groups and were orally administered a placebo or tablets containing 100 mg of *L. gasseri* OLL2809 per d for 8 weeks during the pollen season in 2007. The results showed no obvious differences between the groups. Supplementary subgroup analysis revealed that the OLL2809 subgroups with CAP-RAST scores of 4 or 5 exhibited improvement in nasal symptoms scores and serum allergy-related items, including Japanese cedar pollen-specific IgE levels. *L. gasseri* OLL2809 was found to be effective in reducing symptoms in subjects with a high predisposition to allergies by modulating systemic immune systems.

Key words: Japanese cedar pollen; Japanese cedar pollinosis; *Lactobacillus gasseri*; probiotics; Th1/Th2

In the past few decades, the incidence of Japanese cedar pollinosis (JCP) has been increasing, and a current paper suggests that at least one-sixth of the Japanese population is affected by this allergic disease.[1] Thus far, several medicines, including antihistamines, leukotriene inhibitors, anti-inflammatory cytokines, and corticosteroids, have been developed that greatly contribute to reduction of the symptoms of allergic disease.[2–7] However, because allergic diseases are chronic, continuous treatment is required, and the cost spent on treatment is enormous. In addition, most of the available treatments are symptomatic.[8] Therefore, in view of the high prevalence of JCP and the adverse effects that accompany long-term treatment,[4,9] more effective treatment methods are required.

The increase in the prevalence of allergic diseases has been explained by the hygiene hypothesis proposed by Strachan.[10] Strachan suggested that limited exposure to bacterial and viral pathogens during early childhood leads to insufficient stimulation of the Th1 direction of the immune system and primes an overactive Th2 reaction, leading to allergic disease. The mechanisms underlying this phenomenon are considered to include defective maturation or an absence of regulatory T cells and an inappropriate Th1/Th2 balance.[11] A number of reports suggest correlations between the incidence of allergic diseases and intestinal microbiota, which might serve as stimuli to develop appropriate immune systems.[12,13]

Lactobacilli are gram-positive anaerobic bacteria commensal to humans and animals.[14] For many years, they have been consumed worldwide through foods such as fermented milk. Consequently, they are known to be very safe microorganisms. Moreover, a recent double-blind placebo-controlled clinical study revealed that *Lactobacillus rhamnosus* GG administration suppressed the incidence of atopic diseases in high-risk children by approximately 50%.[15] Hence, the use of lactobacilli might be an easy and effective way to prevent or treating allergies without any side effects.

L. gasseri OLL2809, which was isolated from a human subject, has been selected from approximately 300 *Lactobacillus* strains on the basis of its immunoregulatory effect.[16] We have found using mouse experimental allergy models that when orally administered, heat-killed *L. gasseri* OLL2809 exhibit suppressive effects on antigen-specific IgE and eosinophilia *via* modulation of the Th1/Th2 balance.[16–18] This suggests that heat-killed *L. gasseri* OLL2809 can reduce the clinical symptoms of JCP. In this study, we examined the clinical efficacy of heat-killed *L. gasseri* OLL2809 as to JCP.

Methods

Subjects. Subjects ($n = 107$) aged 20 to 50 years were enrolled in the clinical study. Subjects with JCP who fulfilled the following criteria were enrolled: (i) subjects who experienced JCP symptoms for

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Abbreviations: JCP, Japanese cedar pollinosis; QOL, quality of life; SMS, symptom medication score; IFN, interferon; IL, interleukin

over 2 years, (ii) subjects with serum cedar pollen-specific IgE levels at scores 2 to 5 by the CAP-radioallergosorbent test (CAP-RAST score), (iii) subjects with a moderate total symptom score (comprising symptoms of sneezing, runny nose, nasal congestion, itchy and watery eyes, scored for JCP in the past 2 years) according to the "Practical Guidelines for the Management of Allergic Rhinitis in Japan," 5th edition.[19] Subjects who lived or worked in the suburbs of Tokyo were preferred as study subjects.

The following subjects were excluded from the study: (i) those whose symptoms had developed before the cedar pollen season, (ii) those with nasal disease that might have affected efficacy evaluation in this trial (perennial allergic rhinitis, acute and chronic rhinitis, *etc.*), (iii) those who planned to travel to Hokkaido, Okinawa, or abroad, and (iv) others whom the physician in charge judged unfit as study subjects.

Prior to participation, written consent was obtained from all the subjects after the physician in charge had explained the study to the group. In addition, the study received the approval of the Ethics Committee of the Division of Research and Development of Meiji Dairies Corporation, and it was performed in accordance with the Declaration of Helsinki.

Study design. The study was a randomized, double-blind, placebo-controlled clinical trial performed at a single institution in Tokyo between January 10, 2007 and April 6, 2007. The study protocol is summarized in Fig. 1. After obtaining informed consent from the subjects, they were screened to confirm compliance with the inclusion and exclusion criteria and to examine the physical condition of each individual. Subject background, clinical laboratory analysis (hematology, blood biochemistry, and serology), and physical examination were included in the screening.

Following a 1-week pre-observation period, the subjects were randomly divided into two groups: one group of subjects who were orally administered tablets containing 100 mg (approximately 1×10^{10} cells) of heat-killed (75 °C for 60 min) *L. gasseri* OLL2809 per d and the other, who received placebo tablets. The placebo tablets contained dextrin instead of heat-killed *L. gasseri* OLL2809, and were identical in color and taste to the OLL2809 tablets. Each subject received tablets for an 8-week course (from February 5 to April 6).

On dividing the groups, a controller who was not directly involved in the study was responsible for group allocation. The subjects were divided randomly into the active (OLL2809) and placebo groups according to the total symptom scores for JCP in the past 2 years, the Japanese-cedar-pollen specific IgE not to be different between the groups. A group allocation number was given to each subject. To prevent leakage of information, this number was closely guarded jointly by the controller and a member of the ethical committee who was not directly involved in the study, until accessed with the key after completion of the study.

The physician in charge examined each subject a total of 3 times: during the pre-observation period, and 4 and 8 weeks after treatment. The subjects were asked to fill out the Japanese Allergic Rhinitis QOL standard Questionnaire (JRQLQ) during the pre-observation period, and 4 and 8 weeks after treatment. They were asked to record pollinosis symptoms (sneezing, runny nose, nasal congestion, itchy eyes, watery eyes, and interference with daily life) and compliance with the administration schedule in the subject diary.

Evaluation items.

Nasal cavity findings. Nasal examinations were conducted by rhinoscopy during the pre-observation period and after 4 and 8 weeks of treatment. Mucosal swelling of the inferior turbinate and the amounts of watery rhinorrhea were scored on a 4-point scale of severity (0 = none to 3 = severe).[19]

Allergy diary. Allergy diaries were kept by the subjects to self-assess items from a list, including sneezing (number of attacks per d), runny nose (number of incidences of nose blowing per d), nasal congestion, and itchy and watery eyes. These symptoms were scored subjectively on a 5-point severity scale, where 0 indicated no symptoms; 1, mild symptoms; 2, moderate symptoms; 3, severe symptoms; and 4, very severe symptoms.[19] The total scores for sneezing, runny nose, and nasal congestion were counted as the nasal symptom score, while the total scores for itchy and watery eyes were counted as the ocular symptom score. The medication score was recorded as described elsewhere.[19] Severity during the season was scored daily as nasal or ocular symptom medication scores (SMS), and the mean SMS each week was compared between the placebo and OLL2809 groups.

Japanese allergic rhinitis QOL standard questionnaire (JRQLQ). JRQLQ was used for evaluation of the subjects' QOL during the pollen season. The questionnaire is composed of three parts: nasal and eye symptoms (JRQLQ-I), 17 questions regarding the QOL (JRQLQ-II), and a comprehensive evaluation (face scale).[20]

Nasal and ocular symptoms included the following six categories: runny nose, sneezing, nasal congestion, itchy nose, and itchy and watery eyes. The symptoms of each subject were evaluated on a 5-point scale, 0 denoting no symptoms; 1, mild; 2, moderate to severe; 3, severe; and 4, very severe.

The QOL-related questionnaire included 17 items concerning (i) reduced productivity at work/home; (ii) poor mental concentration; (iii) reduced thinking power; (iv) impaired reading book/newspaper; (v) reduced memory; (vi) limitation of outdoor life (*e.g.*, sports, picnic); (vii) limitation of going out; (viii) hesitation visiting friend or relatives; (ix) reduced contact with friends or others by telephone or conversation; (x) not an easy person to be around; (xi) impaired sleeping; (xii) tiredness; (xiii) fatigue; (xiv) frustration; (xv) irritability; (xvi) depression; and (xvii) unhappiness. Each item was evaluated on a 5-point scale, 0 denoting no significant problem; 1, a mild problem; 2, moderately severe; 3, severe; and 4, very severe.

Blood examination. Blood samples were collected 3 times: at the pre-observation period and after 4 and 8 weeks of treatment. The samples were used to determine the concentrations of total and Japanese cedar pollen-specific IgE levels and number of eosinophils. The ratio of Th1 to Th2 cells (Th1/Th2 ratio) was determined 2 times: in the pre-observation period and after 8 weeks of treatment. The blood examinations described above were performed at SRL (Tokyo).

Assessment of outcomes. The primary efficacy outcome was the difference in the nasal and the ocular symptom medication scores over the 8-week administration period between the OLL2809 and placebo groups. The secondary assessments were based on the QOL-related scores on JRQLQ at 4 and 8 weeks after treatment.

Statistical analysis. A blind data review was performed before decoding, and decisions concerning the handling of drop-outs were made on the basis of blinded results. The assessment of efficacy was based on all the subjects who completed the study. Subgroup analyses based on CAP-RAST scores were done additionally after decoding.

Data were expressed as mean ± SE. Statistical differences between the placebo and OLL2809 groups and subgroups were analyzed by Student's t test or Mann-Whitney's U test. The differences between pre-observation and 4 and 8 weeks after treatment were analyzed in each group and subgroup by Student's paired-t test with Bonferroni's correction. Differences were considered significant when the p-value was less than 0.05.

Results

Background characteristics of subjects and cedar pollen count

Among 107 subjects (placebo $n = 54$, OLL2809 $n = 53$) enrolled, seven subjects withdrew from the study citing personal reasons and 100 (placebo $n = 53$, OLL2809 $n = 47$) successfully completed it. The baseline characteristics of the subjects were similar in the placebo and OLL2809 groups in terms of age, sex, duration of JCP, nasal cavity findings, nasal and ocular symptom medication scores in the allergy diary, and a blood examination (data not shown).

The Japanese cedar pollen season started on January 31 and continued to the end of April in 2007. The total pollen count during the study period was 1,263 grains/cm^2 in Chiyoda-ku, central Tokyo, slightly higher than the pollen count for 2006 (874 grains/cm^2)

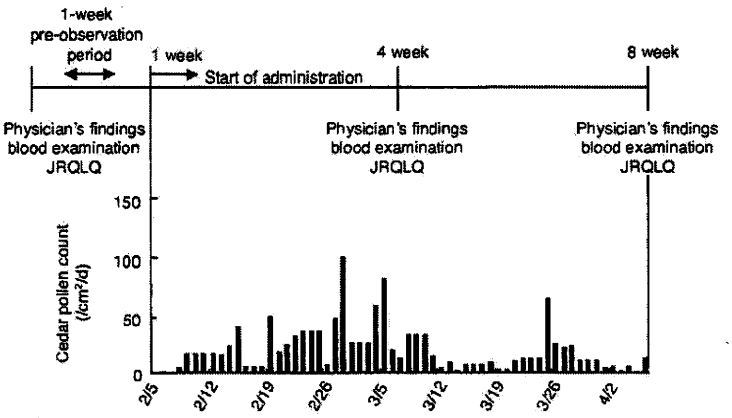

Fig. 1. Study Protocol and Diagrammatic Profile of Japanese Cedar Pollen Dispersion during the Clinical Trial in Tokyo, in 2007.
These data were obtained from the Bureau of Social Welfare and Public Health, Tokyo Metropolitan Government, by determining the pollen collected in a Durham pollen catcher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

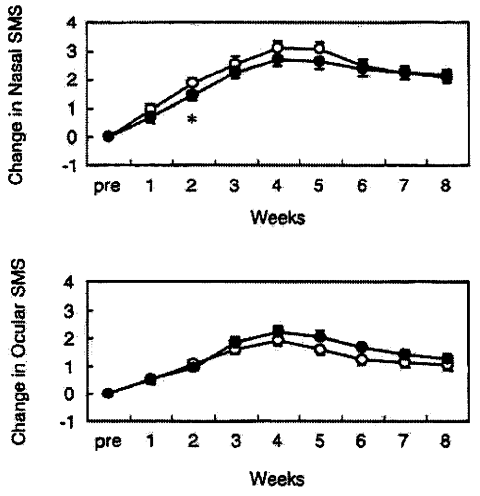

Fig. 2. Time-Course Change in Nasal and Ocular Symptom Medication Scores (SMS).
The scores are differences from those in the pre-observation period. Placebo group (○), $n = 53$; OLL2809 group (●), $n = 47$. $^*p < 0.05$ (Mann-Whitney's U test).

(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, $n = 12$.

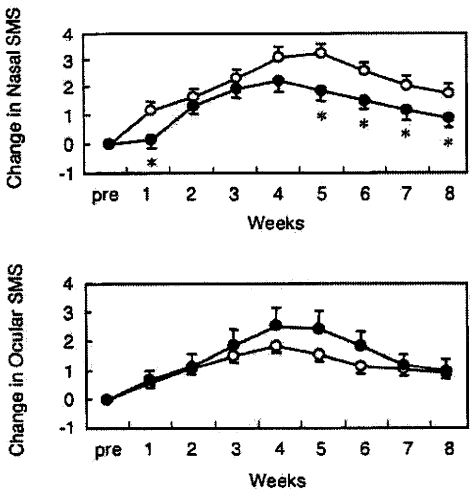

Fig. 3. Time-Course Change in Nasal and Ocular Symptom Medication Scores (SMS) of Subgroups with CAP-RAST Scores of 4 or 5.
The scores are differences from those in the pre-observation period. Placebo subgroup (○), $n = 19$; OLL2809 subgroup (●), $n = 12$. $^*p < 0.05$ (Mann-Whitney's U test).

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

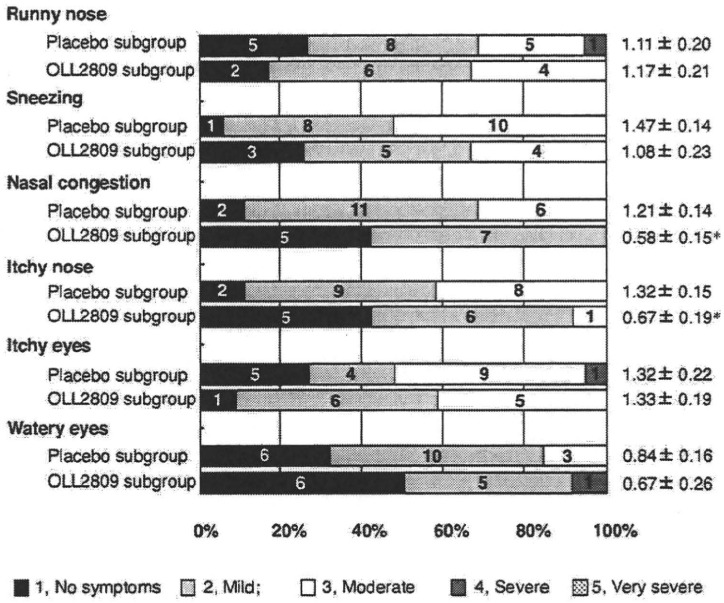

Fig. 4. Japanese Allergic Rhinitis Standard Quality of Life Questionnaire for Subgroups with CAP-RAST Scores of 4 or 5 after 8 Weeks of Treatment.

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

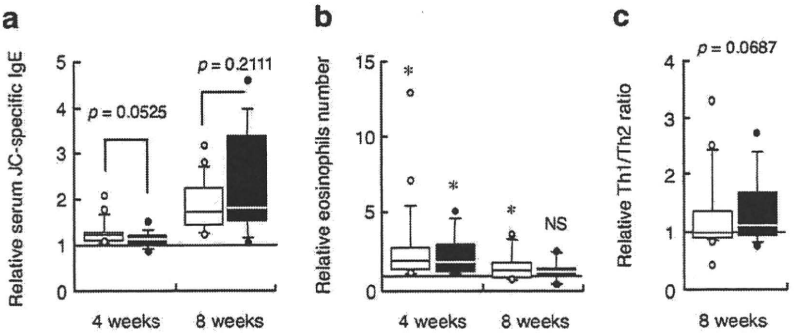

Fig. 5. Relative Changes in Serum Japanese Cedar Pollen-Specific IgE (a), Number of Peripheral Blood Eosinophils (b), and the Th1/Th2 Ratio (c) in Subgroups of Subjects with CAP-RAST Scores of 4 or 5.

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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(総合)研究報告書

スギ抗原免疫療法の候補ペプチドおよび末梢血T細胞のペプチド反応性と制御性T細胞に関する研究

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研究要旨
既知のスギ抗原ペプチド以外の分子を検討し、p206-225 と p61-80 の２つの HLA-DP5 拘束性ペプチドが高い免疫応答を示した。これらはペプチド免疫療法の候補となる可能性が示唆された。また、精製抗原特異的 Th2（IL-4/IL-5）細胞頻度に対するペプチドのそれは症例ごとに異なっていることが ELISPOT 法にて確認され、約 30ー80%程度であった。免疫療法の有効性を高めるには多くの患者が高反応性を示す抗原の開発が必要で、今回の測定系の有用性が示唆された。さらに、スギ抗原特異的制御性 T 細胞（nTreg）の存在を患者末梢血中に確認でき、nTreg は Th1 優位にサイトカイン産生の抑制を行っている可能性が明らかになった。今後、免疫療法における nTreg の変動について検討を進める予定である。

A. 研究目的

スギ花粉抗原である Cry j 1 及び Cry j 2 由来の T 細胞エピトープが同定され、これらを使ったポリペプチドの有用性が in vitro で示されている。
今回の研究は、１）スギ花粉症に対するペプチド免疫療法における既知のペプチド以外の候補分子の可能性を検討すること、２）スギ花粉精製抗原と既知のペプチドの免疫原性の違いをスギ花粉症患者末梢血中の特異的 Th2 細胞の頻度を指標に検討すること、さらに３）Th1/Th2 バランスにおけるスギ抗原特異的 CD4+CD25+制御性 T 細胞（nTreg）の機能を解析すること、を目的とした。

B. 研究方法

１）12 名のスギ花粉症患者より採血。単核球を分離し Cry j 1 存在下に 96 well plate にて 7 日間培養した。さらに APC 存在下に 7 日間培養し T 細胞株を誘導した。誘導された Cry j 1 特異的 T 細胞株を用いて、37 種類の Cry j 1-derived overlapping peptides に対する増殖能を検討した。同時に、この T 細胞からの IFN-・及び IL-4 の産生を ELISA 法によって検討した。
２）5 名のスギ花粉症患者を対象に、末梢血より単球を分離して DC を誘導。Cry j 1 および Cry j 2 または主要 7 種類のエピトープペプチドを誘導した DC に添加して培養。さらに、患者の T 細胞に抗原呈示をさせ ELISPOT 法にて抗原あるいはペプチド特異的 IL-4、IL-5 産生細胞の頻度を測定した。
３）スギ花粉症患者 12 名および健常者 3 名よりリンパ球を分離した。CD4+T 細胞（nTreg 含有群）と CD4+CD25-T 細胞（nTreg 除去群）に分け、これらを Cry j 1 および HLA-DP5 拘束性 Cry j 1 関連ペプチド（p61-75）で刺激し、APC と共に 6 日間培養した。抗原特異的増殖能とサイトカイン産生をそれぞれフローサイトメトリおよび ELISA 法にて測定し両群を比較検討した。また、Cry j 1 特異的 IL-10 産生 Treg の検出を ELISPOT 法にて行った。
（倫理面への配慮）
山梨大学医学部倫理委員会で承認の得られた同意説明文書を被験者に渡し、文書および口頭による十分な説明を行い、試験参加希望者の自由意志による試験への参加について同意を文書で受け取った。同意説明書には予期される副作用と効果、試験への参加は任意であることと同意しない場合ことをもって不利益な対応は受けないこと、参加の同意はいつでも撤回できること、試験に伴う補償の有無、個人情報の取扱いと関連する手続きなどの内容が含まれている。

C. 結果

１）スギ花粉症全員から Cry j 1 特異的 T 細胞株を誘導できた。これらは Cry j 1 タンパクに対し増殖能を示しかつ IFN-・あるいは IL-4 を産生したがその産生量は様々であった。

誘導された Cry j 1 特異的 T 細胞株の overlapping peptides37 種類に対する増殖能の検討では、15 種類のペプチドが少なくとも 1 つの T 細胞株を刺激できた。特に 4 つのペプチド、p61-80（3/12; 25.0%）、p115-132（2/12; 16.6%）、p206-225（4/12;

33.3%)、p337-353 (5/12; 41.7%) は1つ以上のT細胞株に対し増殖反応を誘導した。逆に、12名中11名はこれら4つのペプチドの少なくとも1つに反応を示した。9例のT細胞株でCry j 1に対するサイトカイン産生を評価し、2例はTh2優位 (IL-4)、3例はTh1優位 (IFN-・)、4例はTh0パターン (IFN-・とIL-4が同程度) を示し様々であった。

overlapping peptidesに対するサイトカイン産生はCry j 1タンパクに対するそれと同じではなかった。2例から得られたT細胞株はそれぞれp86-105、p271-290のペプチドに対してIFN-・のみを産生した。

2)ペプチド特異的IL-4・IL-5産生細胞とCry J 1,2特異的IL-4・IL-5産生細胞との間には高い正の相関が見られた。ペプチド特異的IL-4産生細胞の頻度はCry J 1,2特異的IL-4産生細胞のそれの65.5ー32.6%の割合であり、症例毎に異なっていた。同様に、ペプチド特異的IL-5産生細胞の頻度はCry J 1,2特異的IL-5産生細胞のそれの81.6ー29.6%の割合であり、症例で異なっていた。3)細胞増殖能にはCry j 1およびCry j 1ペプチド (p61-75) 刺激いずれにおいても両群間で差は認めなかった。IFN-・産生に関してはCry j 1刺激でnTreg除去によりその産生は有意に増加したが、IL-5産生に関しては、nTreg除去の影響は認めなかった。また、IL-10の産生はCry j 1刺激においてnTregの除去により有意に低下した。

3名のスギ花粉症患者において、Cry j 1特異的CD4+CD25+ (nTreg) IL-10産生細胞をELISPOTにて確認できた。

D. 考察:

1) これまでCry j 1由来ペプチドとして、4種類 (p108-120, p211-225, p235-247, p312-330) が選ばれpolypeptideとして合成されている。我々の研究でもp206-225において33.3% (4/12) で免疫応答を示した。一方、p61-80とp337-353はそれぞれ25%、41.7%と比較的高い免疫応答を示した。p214-222、p61-80は日本人で比較的多いHLA-DP5拘束性に提示されることが示されており、p206-225とp61-80は高率に反応を示したのかも知れない。ゆえにこれら2つのペプチドはペプチド免疫療法の良い候補分子となる可能性が示唆された。2)ペプチド特異的Th2産生細胞とCry J 1,2特異的Th2産生細胞との間には相関があり、ペプチドを用いた場合にもある程度その免疫原性は保たれている可能性があると思われる。しかしながら、7種類のペプチドに限定すると、反応性が高い患者と低い患者が存在することが明らかとなり、実際の治療に用いるためには、より多くの患者がカバーできかつ高い反応性を持ったペプチドの選択が必要であると考えられた。3)スギ花粉症患者においては、nTregはTh2よりもTh1優位に抑制機構が働いていることがわかった。また、スギ花粉症患者の末梢血にはCry j 1特異的IL-10産生Tregが存在し、スギ抗原特異的免疫応答の制御に関与している可能性が示唆された。

E. 結論

1) 我々の研究で示した4種類のペプチドは免疫応答が比較的高率に認められ、ペプチド免疫療法のペプチドデザインに有用である可能性が示唆された。

2) 有効なペプチド免疫療法普及には安全かつ効果的でコストバランスに優れたものが必要である。今後新たな抗原を開発する上で従来の精製抗原との免疫原性の比較検討は重要な課題であり、今回用いた測定系は有用であった。

3) スギ抗原特異的制御性T細胞 (nTreg) の存在を確認した。また、その機能は主にTh1優位に抑制している可能性が示唆された。

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G. 知的財産権の出願・登録状況（予定を含む）

１．特許取得

なし

２．実用新案登録

なし

３．その他

なし

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SHORT COMMUNICATION

TGF-β Signaling May Play a Role in the Development of Goblet Cell Hyperplasia in a Mouse Model of Allergic Rhinitis

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ABSTRACT

Background: Transforming growth factor-β (TGF-β) levels are elevated in the nasal mucosa in allergic rhinitis. However, because TGF-β is secreted extracellulary in latent complexes, it remains unclear whether the local TGF-β expression actually drives active signaling and affects the pathophysiology of allergic rhinitis. The objective of this study is to investigate whether TGF-β signaling is activated in allergic rhinitis and plays a role in the pathophysiology of allergic rhinitis.

Methods: An ovabumin (OVA)-sensitized and -nasally challenged mouse model of allergic rhinitis was established and phosphorylation of Smad2 in the nasal mucosa was examined by immunohistochemistry. In addition, the effects of the pharmacological inhibition of endogenous TGF-β signaling on the allergic rhinitis model were histologically examined. Furthermore, phosphorylation of Smad2 in the nasal mucosa samples obtained from patients with allergic rhinitis was also evaluated.

Results: In the mouse model of allergic rhinitis, OVA challenge induced phosphorylation of Smad2 predominantly in epithelial cells in the nasal mucosa. In addition, the administration of an inhibitor of TGF-β type I receptor kinase activity during OVA challenge suppressed goblet cell hyperplasia in the nasal mucosa. Furthermore, phosphorylated Smad2 expression increased in nasal epithelial cells in patients with allergic rhinitis.

Conclusions: These results suggest that TGF-β signaling is activated in epithelial cells in the nasal mucosa in allergic rhinitis and may contribute to the development of goblet cell hyperplasia.

KEY WORDS

allergic rhinitis, epithelial cells, Smad, TGF-β

ABBREVIATIONS

OVA, ovalbumin.

INTRODUCTION

Allergic rhinitis (OMIM #607154) is a common chronic disease of the nasal mucosa. Over 10% of the population in developed countries suffers from allergic rhinitis, which creates societal burdens due to such factors as increased medical expenses and a loss of productivity.[1,2] Allergic rhinitis is pathologically characterized by Th2-type allergic inflammation, including eosinophil infiltration, goblet cell hyperplasia, and mast cell accumulation in the nasal mucosa.[3]

TGF-β is a multifunctional cytokine that regulates cell growth, differentiation, and survival, belonging to a large family of structurally related proteins, known as the TGF-β family, to which also activins and bone morphogenetic proteins (BMPs) belong.[4] TGF-β fam-

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ily ligands bind to two different types of serine/threonine kinase receptors, termed type I and type II. Type I receptor is activated by type II receptor upon ligand binding and transduces signals principally through the Smad family of proteins.[5] Smad2 and Smad3 are phosphorylated by activated TGF-β and activin type I receptors whereas Smad1, Smad5, and Smad8 are phosphorylated by activated BMP type I receptors.[5,6] Several small molecule inhibitors of TGF-β type I receptor kinase activity have been recently developed and are considered to be a promising reagent for the treatment of cancer and fibrotic diseases.[7]

In patients with allergic rhinitis, TGF-β protein expression is significantly increased in the epithelial cells in the nasal mucosa.[8] However, because TGF-β is secreted extracellulary as latent complexes and thus requires activation to mediate its effects,[9] the actual activity of TGF-β in the nasal mucosa of allergic rhinitis and its roles in the pathophysiology of allergic rhinitis remain uncertain.

In this study, we assessed the activation of TGF-β signaling and its roles in allergic rhinitis using a mouse model of allergic rhinitis and the nasal mucosa specimens derived from patients with allergic rhinitis by the detection of phosphorylation of Smad2 and by the pharmacological inhibition of endogenous TGF-β signaling.

METHODS

MICE

Female 4-6 wks BALB/c mice were purchased from Japan SLC (Tokyo, Japan) and were bred under specific pathogen-free conditions.

ALLERGIC RHINITIS MODEL

An allergic rhinitis model was established as previously described with some modifications.[10] Briefly, the mice were actively immunized i.p. with 10 μg of ovalbumin (OVA, Sigma Aldrich, St. Louis, MS, USA) in 4 mg of aluminum hydroxide on Day 0 and Day 7. Starting on Day 14, they were challenged intranasally with 100 μg OVA in 10 μl PBS twice per day for 1 week (total 14 times/week). The mice were challenged intranasally with PBS in a similar manner for the negative control. For some experiments, HTS 466284 (10 mg/kg) (Calbiochem, San Diego, CA, USA)[11] or a control vehicle (DMSO) was intraperitoneally administered every other day, starting on Day 14 until sacrifice. The dosage of HTS466284 (10 mg/kg) was based on previous experiments.[12] The animal experiments were approved by the Institutional Review Board of the University of Yamanashi.

HISTOLOGY

Twelve hours after the final nasal challenge, mice were killed with carbon dioxide. The heads were removed, fixed, and decalcified. Coronal nasal sections were visualized by staining with hematoxylin and eosin (HE) or Hansel staining (to demonstrate eosinophils), or periodic acid-Schiff (PAS)/hematoxylin (to demonstrate goblet cells).

IMMUNOHISTOCHEMISTRY

To detect phosphorylated Smad1 and Smad2, the coronal nasal sections were deparaffinized and stained with anti-phosphorylated Smad2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or anti-phosphorylated Smad1 antibody[13] through the use of peroxidase-based VECTASTAIN ABC kits with DAB substrate (Vector Laboratories, Burlingame, CA, USA). Nuclei were counter-stained with hematoxylin. The sections were photographed by digital color-CCD camera (BX50, Olympus, Tokyo, Japan).

QUANTIFICATION OF HISTOLOGICAL EXAMINATION

The number of phosphorylation of Smad2-positive cells in the nasal sections was counted as previously described.[14] Briefly, a minimum of 500 cells in the nasal epithelium was counted in at least 6 high power fields (×400) in each sample. The percentage of phosphorylated Smad2-positive cells in the total nasal epithelial cells was expressed (%) and the mean percentage was calculated in 6 animals or 4 human samples. The number of infiltrating eosinophils in the nasal mucosa and PAS-positive goblet cells in the nasal mucosa in the posterior portion of nasal septum was determined microscopically in a blinded manner and expressed as the number per high-power field (400×). Two or four specimens of the Hansel- or PAS-stained coronal sections from one mouse were selected. The mean score was counted, and then the mean scores were calculated in 6 animals.

IMMUNOFLUORESCENE

For phosphorylated Smad2 (pSmad2) labeling, the coronal nasal sections were blocked for 10 minutes in 3% H_2O_2, incubated with rabbit anti-pSmad2 antibody (Santa Cruz Biotechnology Inc., 1 : 200 in 1% BSA, 2 hours at room temperature) and then incubated with swine anti-rabbit antibody conjugated to RITC (red) (1 : 20 in PBS, 40min) (DAKO Cytomation, Glostrup, Denmark). The pictures were taken on an Olympus fluorescent microscope (DP30BW, Olympus, Tokyo, Japan).

BIOPSY SAMPLES

Inferior turbinate thiny biopsies of 4 seasonal allergic rhinitis patients were obtained using a cup forceps device under local anesthesia. Control biopsy samples were obtained from 4 patients with idiopathic maxillary cyst during the surgical operations. The control patients had no history of allergic diseases at the operations. Informed consent for the described

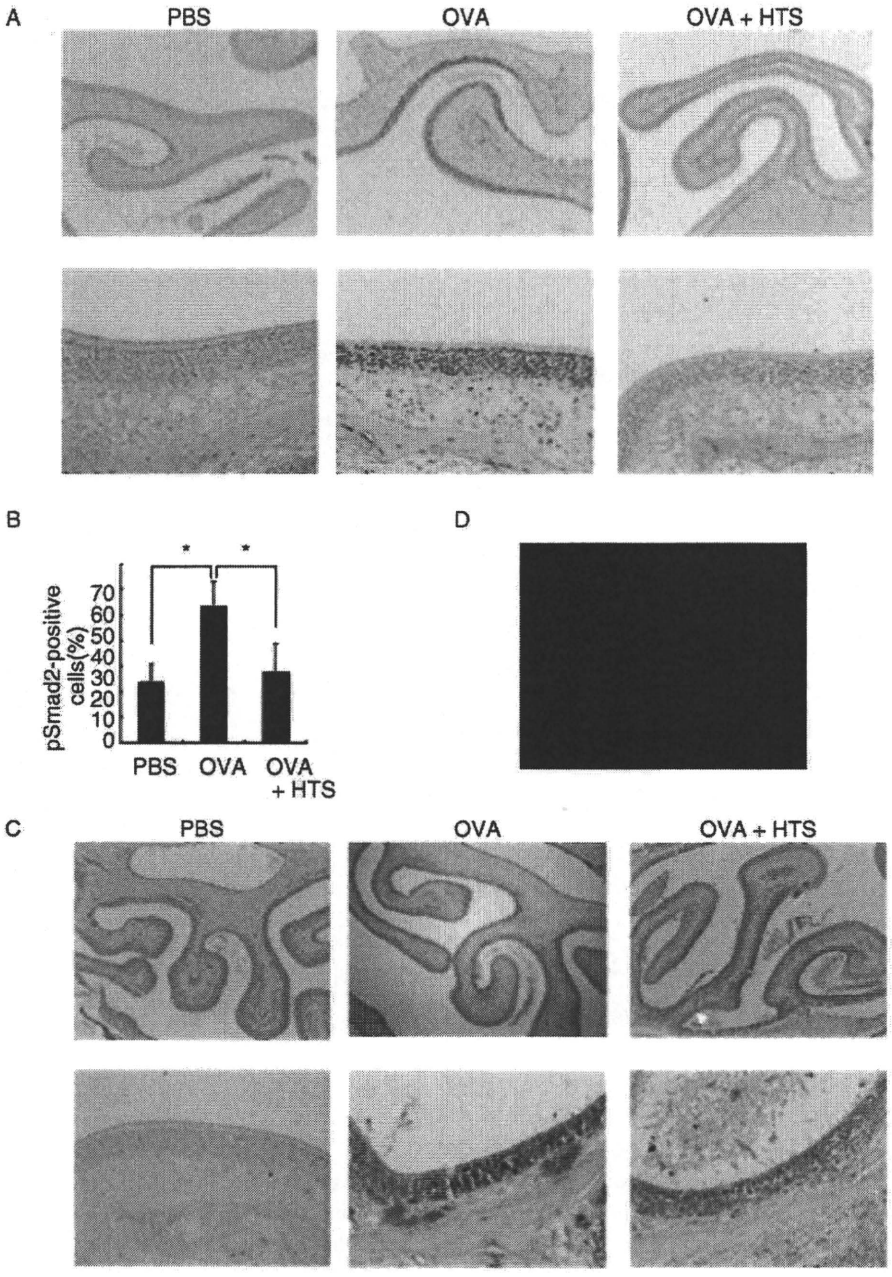

Fig. 1 Phosphorylation of Smad2 was detected predominantly in epithelial cells in the nasal mucosa in a mouse model of allergic rhinitis. The OVA-sensitized mice were intranasally challenged with OVA or PBS. During the OVA challenge, HTS466284 (OVA + HTS) or the control vehicle DMSO (OVA) was administered intraperitoneally every other day. PBS: OVA-sensitized and PBS-challenged control mice. **A-C.** Representative pictures (upper panels: ×40, lower panels: ×400) of immunohistochemical staining with anti-phosphorylated Smad2 antibody (**A**) or anti-phosphorylated Smad1 antibody (**C**) and a quantitative analysis of the epithelial phosphorylated Smad2 expression in the model of allergic rhinitis (**B**). Positive staining indicates as brown. **D.** Immunofluorescent analysis with anti-phosphorylated Smad2 antibody (red). Representative pictures of the nasal mucosa obtained from the OVA-sensitized and -challenged mice treated with control vehicle (DMSO) are shown. Positive staining indicates as red. Values represent the mean ± SD of 6 mice in each group. $^*p < 0.05$.

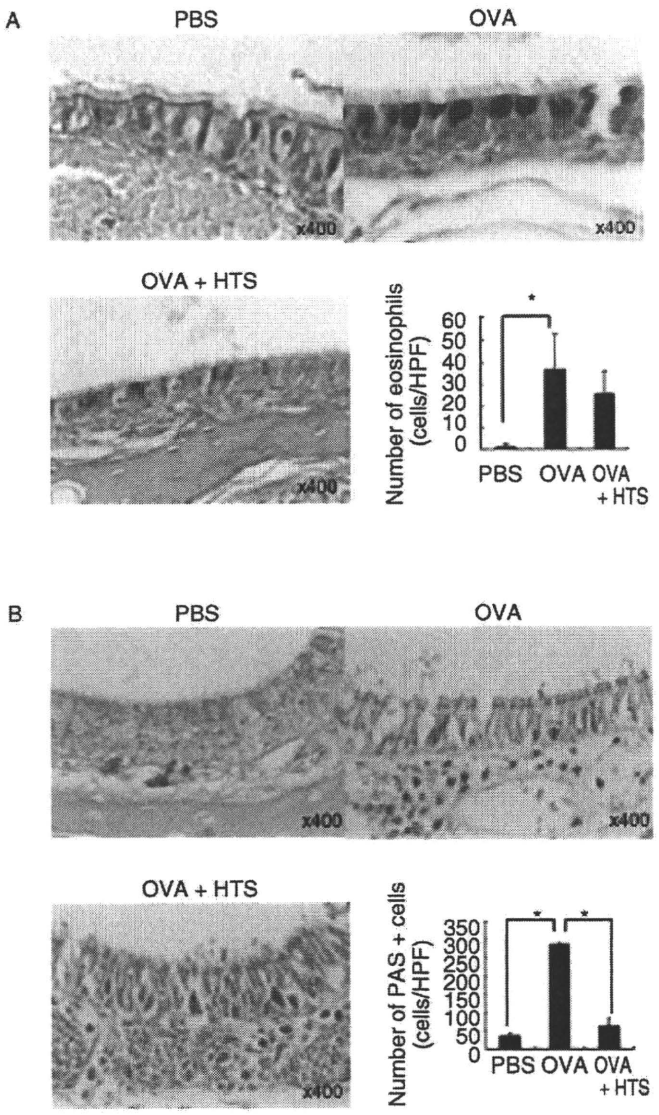

Fig. 2 TGF-β signaling may contribute to the development of goblet cell hyperplasia in the nasal mucosa in a mouse model of allergic rhinitis. The allergic rhinitis model was established as described in Figure 1 legend. **A-B.** Representative picture of Hansel (**A**) and PAS (**B**) staining of the nasal mucosa obtained from the mice treated with HTS466284 (OVA + HTS) or control vehicle DMSO (OVA) or OVA-sensitized and PBS-challenged control mice (PBS). Bar graphs show quantitative analysis of the number of eosinophils in the nasal mucosa and PAS-positive goblet cells in the nasal epithelium. Values represent the mean ± SD of 6 mice in each group. $*p < 0.05$.

procedure was obtained from all patients. Approval for the study was given by the ethics committee of the University of Yamanashi. The specimens were fixed in 4% paraformaldehyde for 3 hours and then embedded in paraffin. The number of phosphorylated Smad2-positive cells in the nasal sections was counted as described above.

STATISTICAL ANALYSIS

The data are summarized as the mean ± SD. Statistical analysis was performed using the non-parametric Mann-Whitney *U* test to compare data in different two

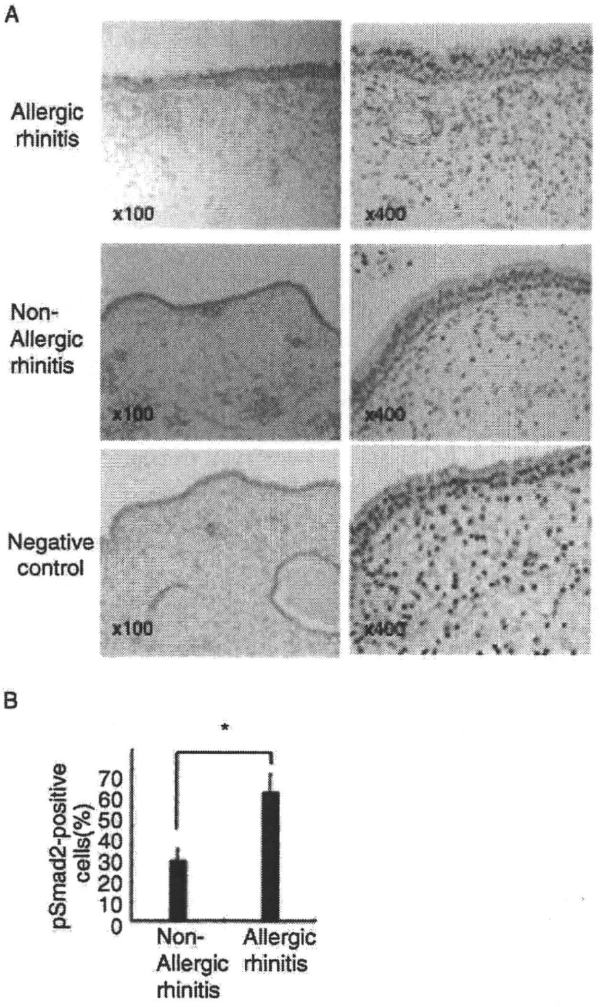

Fig. 3 Phosphorylation of Smad2 was detected predominantly in epithelial cells in the nasal mucosa in patients with allergic rhinitis. **A.** Four allergic rhinitis or 4 non-allergic rhinitis tissue specimens were immunohistochemically stained with anti-phosphorylated Smad2 antibody. Representative pictures are shown. Negative control: allergic rhinitis tissue specimens stained with control rabbit IgG antibody. **B.** A quantitative analysis of the epithelial phosphorylated Smad2 expression in allergic rhinitis and non-allergic rhinitis specimens ($n = 4$ in each group). $^*p < 0.05$.

groups. A value of $P < 0.05$ was considered to be significant.

RESULTS

PHOSPHORYLATION OF SMAD2 WAS DETECTED PREDOMINANTLY IN EPITHELIAL CELLS IN THE NASAL MUCOSA IN A MOUSE MODEL OF ALLERGIC RHINITIS

To determine whether TGF-β signaling is active in allergic rhinitis, we examined the phosphorylation of Smad2 in the nasal mucosa in a mouse model of allergic rhinitis because phosphorylation of Smad2 is a key event for initial TGF-β signal transduction.[5]

Immunohistochemical staining for phosphorylated Smad2 revealed the immunoreactivity to be increased predominantly in the nasal epithelium and in some submucosal cells after the induction of allergic rhinitis (Fig. 1A, B). Interestingly, we also found that immunoreactivity for phosphorylated Smad1, a key indicator for initial BMP signaling, also increased predominantly in the nasal epithelium (Fig. 1C).

Consistent with the immunohistochemical findings, an immunofluorescence staining also confirmed the phosphorylated Smad2-positive cells to be present predominantly in the nasal epithelium (Fig. 1D). These results suggested that epithelial cells predominantly received endogenous TGF-β activity in the nasal mucosa in a mouse model of allergic rhinitis.

ACTIVATION OF TGF-β SIGNALING MAY CONTRIBUTE TO THE DEVELOPMENT OF GOBLET CELL HYPERPLASIA IN A MOUSE MODEL OF ALLERGIC RHINITIS

Because active TGF-β signaling was present in the nasal mucosa in a mouse model of allergic rhinitis (Fig. 1), we determined whether activation of TGF-β signaling plays some roles for the development of the allergic rhinitis model. For this purpose, the effects of TGF-β type I receptor kinase inhibitor[11] HTS466284 on the development of allergic rhinitis were pathologically evaluated. The administration of HTS 466284 during OVA challenge significantly inhibited phosphorylation of Smad2, but not Smad1, in the nasal epithelium (Fig. 1A, C), suggesting that the inhibitor was indeed specific to TGF-β signaling.

OVA-sensitized BALB/c mice treated with control vehicle (DMSO) during OVA challenge showed massive infiltration of eosinophils into the nasal mucosa and increased number of PAS-positive goblet cells in the nasal mucosa (Fig. 2A, B). OVA-sensitized mice treated with HTS466284 during OVA challenge showed marginal reduction of the infiltration of eosinophils into the nasal mucosa (Fig. 2A). Importantly, the number of PAS-positive goblet cells in the nasal mucosa decreased to the basal levels in HTS 466284-treated mice (Fig. 2B). These results suggested that the pharmacological blockade of endogenous TGF-β signaling inhibited the development of goblet cell hyperplasia in the nasal mucosa in a mouse model of allergic rhinitis without affecting eosinophil infiltration into the nasal mucosa.

PHOSPHORYLATION OF SMAD2 WAS DETECTED PREDOMINANTLY IN EPITHELIAL CELLS IN THE NASAL MUCOSA IN PATIENTS WITH ALLERGIC RHINITIS

Finally, the relevance of the findings in mice to humans was investigated. The nasal mucosa specimens derived from 4 patients with allergic rhinitis showed an increase in the number of phosphorylated Smad2-

postive cells in nasal epithelium when compared with that in non-allergic rhinitis subjects (Fig. 3A, B). These results suggested that epithelial cells predominantly received endogenous TGF-β activity in the nasal mucosa in patients with allergic rhinitis.

DISCUSSION

A previous study using immunohistochemistry showed significantly increased immunoreactivity for TGF-β in the epithelial layer, with predominant localization to the superficial columnar epithelial cells, of the nasal mucosa obtained from patients with allergic rhinitis.[8] However, because TGF-β is secreted extracellular as latent complexes, it remains unclear whether the local TGF-β expression actually drives active signaling and, if any, what roles the active TGF-β signaling play in allergic rhinitis. In this study, we suggest that active TGF-β signaling is present in the nasal mucosa of allergic rhinitis and it may play a role in the development of goblet cell hyperplasia in allergic rhinitis.

Phosphorylation of Smad1 as well as that of Smad2 was detected in the nasal epithelium in the allergic rhinitis model (Fig. 1C). Because Smad1 principally mediates BMP signals,[5,6] these results suggest that endogenous BMP signaling may be also involved in the pathophysiology of allergic rhinitis. In a mouse model of asthma and in human asthmatics, BMP signaling was reported to be activated upon allergen provocation in the airway epithelium,[3,15] suggesting that BMP signaling may be involved in the tissue repair and control of inflammation. Thus, active BMP signaling in allergic rhinitis may also play a role in these processes. The precise roles of BMP signaling in allergic rhinitis as well as in asthma remain to be determined.

TGF-β has been implicated in the regulation of airway mucin production.[16] For instance, TGF-β increased mucin MUC5AC protein expression in cultured human bronchial epithelial cells[17] and neutralization of TGF-β activity using anti-TGF-β antibody or a TGF-β type I receptor kinase inhibitor suppressed antigen-induced increase in PAS-positive cells in mouse models of asthma.[18,19] In addition, Smad3-deficient mice developed a significantly reduced percentage of airway epithelium that stained positive with PAS in a model of asthma when compared with wild type mice.[20] Taken together with our current *in vivo* findings, it is very likely that TGF-β signaling in allergic rhinitis contributes to the development of goblet cell hyperplasia in the nasal mucosa. Because it remains unclear whether TGF-β signaling exerts its effects either directly or indirectly on nasal epithelial cells, in particular, *in vivo* situations, future studies should focus on the effects of TGF-β signaling on the regulation of goblet cell differentiation, proliferation, and mucin production.

The pharmacological blockade of endogenous TGF-β signaling did not affect the number of eosinophils infiltrated into the nasal mucosa in the allergic rhinitis model (Fig. 2). These results are consistent with the previous findings obtained from mouse models of asthma, showing that the blockade of endogenous TGF-β signaling did not affect airway inflammation.[18,20,21] However, it should be noted that the roles of TGF-β in airway inflammation are still controversial, depending on the models and protocols[22] and thus requires further investigations.

In summary, this study suggests that TGF-β signaling is activated in the nasal mucosa in allergic rhinitis and may contribute to the development of goblet cell hyperplasia in the nasal mucosa in allergic rhinitis. To our knowledge, this is the first report showing that active TGF-β signaling is present in allergic rhinitis and addressing possible roles of TGF-β signaling in the pathophysiology of the disease. Based on the current results, TGF-β signaling in nasal mucosa might become a potential target for the prevention of a selective pathological feature of allergic rhinitis.

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