

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- γ のみを産生した。

D. 考察

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。

一般に、アレルギー患者の T 細胞は Th2 反応を示すことが多い。しかし、実際にはペプチドの長さや性質、その濃度、提示される HLA 分子などによって様々であると言われている。我々の研究でも p86-105、p271-290 に反応した T 細胞株は IFN- γ のみを産生した。Th1 細胞は Th2 機能に対して抑制効果を示すとも言われている。しかしながら、Th1 細胞が制御性 T 細胞とどのように相互関与しているのかについてはよくわかっていない。各々のペプチドの性質と Cry j 1 特異的 T 細胞反応についての更なる検討が、より効果的なペプチド免疫療法の開発のために必要であろうと考えられる。

E. 結論

我々の研究で示された 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。

F. 研究発表

1. 論文発表

- 1) Masuyama K, Chikamatsu K, Ikagawa S, Matsuoka T, Takahashi G, Yamamoto T, and Endo S. Analysis of helper T cell responses to Cry j 1-derived peptides in patients with nasal allergy: candidate for peptide-based immunotherapy of Japanese cedar pollinosis.

Allergol Int 2009;58:63-70.

- 2) Chikamatsu K, Sakakura K, Matsuoka T, Endo S, Takahashi G, Matsuzaki Z, and Masuyama K. Analysis of T-helper responses and FOXP3 gene expression in patients with Japanese cedar pollinosis. Am J Rhinol 2008;22:582-588.
- 3) Takahashi G, Matsuzaki Z, Nakayama T, and Masuyama K. Patterns of drug prescription for Japanese cedar pollinosis using a clinical vignette questionnaire. Allergol Int 2008;57:405-411.
- 4) 増山敬祐. アレルギー疾患におけるステロイドの使い方 鼻噴霧用ステロイド薬の使い方 コツと落とし穴. アレルギー 2009;58:620-629.

2. 学会発表

- 1) 松岡伴和、増山敬祐：スギ抗原特異的メモリー T (IL-4, IL-5, IL-10) 細胞の季節変動について. 第 27 回日本耳鼻咽喉科免疫アレルギー学会、2009.
- 2) 高橋吾郎、松崎全成、増山敬祐：スギ花粉症に対する初期療法に関するランダム化比較試験—RQLQ 日本語版による QOL の評価—. 第 27 回日本耳鼻咽喉科免疫アレルギー学会、2009.
- 3) 上條篤、高橋吾郎、松岡伴和、松崎全成、増山敬祐：スギ花粉症に対するボツリヌス治療の有効性の検討 (E-BOAT STUDY). 第 59 回日本アレルギー学会、2009.
- 4) 松岡伴和、高橋吾郎、松崎全成、増山敬祐：スギ花粉症患者に対する QOL 調査 特異的免疫療法の効果を中心に. 第 48 回日本鼻科学会、2009.

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

Patterns of Drug Prescription for Japanese Cedar Pollinosis Using a Clinical Vignette Questionnaire

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ABSTRACT

Background: Although prescribed drugs directly affect patient outcome, the variation in physicians' attitudes towards drug therapy for cedar pollinosis has not been quantitatively assessed. This research investigated the prescription patterns of drugs for cedar pollinosis by ear, nose, and throat specialists (ENTs), general physicians (GPs) and internal medicine doctors (IMs) in Yamanashi Prefecture, Japan.

Methods: A cross-sectional study was conducted by mailing questionnaires to 532 physicians in autumn 2006. The main part of the questionnaire constituted clinical vignettes of pollinosis cases with nasal and ocular symptoms ranging from mild to severe. We requested that the physicians fill out prescription medications they considered appropriate for each vignette.

Results: Responses from 172 physicians (32%) for six clinical vignettes were analyzed. The number of drugs prescribed by ENTs was significantly higher than that by GPs and IMs for vignettes representing moderate to severe cases ($p < 0.004$). The percentage of physicians who said they would prescribe nasal corticosteroid and eye drops was higher in the ENT group compared to the other two groups in these vignettes. In terms of second-generation antihistamines, no differences were observed between the three groups for all vignettes.

Conclusions: Our investigation suggested that, compared to ENTs, GPs and IMs have a lower tendency to concomitantly prescribe drugs for localized treatment such as nasal corticosteroids and eye drops with oral medication. There may be differences in prescription patterns of drugs for pollinosis between ENTs and non-specialist physicians.

KEY WORDS

allergic rhinitis, guideline, Japan, physician's practice patterns, questionnaires

INTRODUCTION

Allergic rhinitis is not a life-threatening illness, nevertheless patients suffer from highly uncomfortable symptoms that disrupt the quality of everyday life and productivity of academic or professional work.¹ Symptoms of cedar pollinosis begin to appear around February to March every year, and it is the most common type of seasonal allergic rhinoconjunctivitis in Japan. According to a nationwide epidemiological study, the prevalence of Japanese cedar pollinosis is estimated to be 13%. However, recent studies based on statistical analyses predict potential annual increases in this figure.^{2,3} Such high figures demon-

strate that Japanese cedar pollinosis is indeed a large problem in society.

In recent years, variation in the medical practice of physicians has been the subject of research across many clinical fields, from the perspective of the quality of healthcare.^{4,7} When considering the huge impact cedar pollinosis has on society, it is important to research the variations in medical practice for this particular illness, especially with regard to the patterns of drug prescription.

Currently, nasal corticosteroid drops are considered to be the first-line drug for patients with moderate to severe allergic rhinitis.¹ However, a cross-sectional study has suggested that, in actuality, the

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prescription of nasal corticosteroids by general physicians may be limited.⁸ According to Demoly *et al.* whose research involved patients and general physicians, oral antihistamines were prescribed for 92% of the patients, whereas only 45% were prescribed nasal steroids.⁹ Similarly, a patient survey conducted in Japan by Okuda *et al.* revealed a higher tendency of general physicians to singly prescribe oral medication compared to otolaryngologists.¹⁰ Furthermore, Van Hoecke *et al.* have shown with reference to the Allergic Rhinitis and its Impact on Asthma (ARIA) guideline that 30% of medicines prescribed by general physicians for moderate or severe persistent allergic rhinitis patients were considered as undertreatment.¹¹ This is a problem concerning compliance with the guidelines for drug therapy. However, additional research conducted in, these previous studies on clinically prescribed medication that are often influenced by many factors such as the patients' clinical conditions, personal values, healthcare environments, medical resources and annual variance in antigen levels suggest that it is not appropriate to interpret the results simply as patterns of decision-making processes or patterns of drug prescription by individual physicians.

To date, the prescription patterns of drugs for cedar pollinosis have not been investigated. In this study, we have investigated such prescription patterns by Japanese physicians under the hypothesis that general physicians depend less on nasal steroids than otolaryngologists.

Traditionally, this type of research has been conducted using methods such as assessments involving simulated patients, or by reviewing medical records. Recently, the validity and advantages of using clinical vignettes for such research have been shown, and this is now becoming a method of interest.^{12,13}

The aim of this research is to compare the prescription patterns of drugs for cedar pollinosis by ear, nose, and throat specialists (ENTs), general physicians (GPs) and internal medicine doctors (IMs) in Yamanashi Prefecture, Japan.

METHODS

RESEARCH DESIGN, SETTINGS AND SUBJECTS

This research was designed as a cross-sectional study carried out using mailed questionnaires. Subjects were ENTs, GPs and IMs working in Yamanashi Prefecture. The exact number of subjects was unknown at the time of this study, although there were 59 ENTs and 491 physicians (total number of GPs and IMs) in 2004 according to a report by the Ministry of Health, Labour and Welfare.¹⁴

QUESTIONNAIRE DESIGN

The questionnaire was constructed by three ENTs. It consisted of questions regarding 1) occupational

background of the subjects, 2) medical consultation for allergic rhinitis, and 3) clinical vignettes for cedar pollinosis. In principle, questionnaires were answered anonymously and were self-completed. For each vignette, the most effective prescribed medication was decided by the subjects and noted together with any co-administered drugs or required medication.

RESEARCH METHODS

The names and addresses of 53 ENTs, 214 GPs and 265 IMs were found individually by searching through phone books and the Internet. Questionnaires were mailed to them on October 10, 2006. Reminders were sent twice thereafter and the questionnaires were collected by November 10, 2006. This research was conducted upon the approval of the Ethics Review Board of the University of Yamanashi Hospital.

STATISTICAL ANALYSIS

Background factors of the subjects were presented descriptively. Responses for six clinical vignettes were analyzed (Appendix). The names of prescribed medication given for each vignette were categorized and sorted into second-generation antihistamines, oral steroids, antileukotrienes, other oral medication (first-generation antihistamines, Chinese herbal medicines, chemical mediator release inhibitors etc.), nasal corticosteroid drops, non-steroidal anti-allergy nasal drops (antihistamines, chromones etc.), nasal vasoconstrictive agents and eye drops (steroids, antihistamines, chromones etc.). Fexofenadine hydrochlorides and loratadins that were not accompanied with a product leaflet containing information on precautions for vehicular driving were categorized as non-sedative antihistamines, and analysis was carried out accordingly.

The Kruskal-Wallis test was used to compare the number of prescribed medications between the 3 groups of prescribers for each vignette. The significance level was used according to the method by Bonferroni at $\alpha = 0.008$ (two-tailed). This test was carried out for the null hypothesis: there was no difference in the number of prescribed medications between the 3 groups. For vignettes where the null hypothesis was rejected, analysis was repeated using a Mann-Whitney test between the 2 groups using a two-tailed significance level of $\alpha = 0.004$, again using the Bonferroni method.

Furthermore, for each type of drug and for each vignette, both the percentage of physicians who prescribed the drug and its 95% confidence intervals (or one-sided 97.5% confidence interval) were used to compare the 3 groups.

All statistical analyses were performed in Stata version 9.2 (StataCorp, College Station, Tx, USA).

Table 1 Characteristics of physicians included in the survey

	ENT <i>n</i> = 45	GP <i>n</i> = 72	IM <i>n</i> = 55
Male, no. (%)	39 (87)	68 (94)	48 (87)
Years since graduation from medical school, median (IQR)	20 (14–27)	25 (20–33)	13 (8–20)
Physicians with a solo practice, no. (%)	24 (53)	56 (78)	3 (5)
Greatest number of pollinosis patients per day examined in the Japanese cedar pollinosis season of 2006, no. (%)			
1–10	7 (16)	0 (0)	42 (76)
11–30	16 (36)	45 (63)	10 (18)
31–50	3 (7)	25 (35)	2 (4)
51–	19 (42)	1 (1)	1 (2)
Unknown	0 (0)	1 (1)	0 (0)
Usefulness of Japanese practical guideline for AR in the Japanese cedar pollinosis season of 2006, no. (%)			
Very useful	12 (27)	11 (15)	11 (20)
Useful	27 (60)	51 (71)	27 (49)
Neutral	5 (11)	2 (3)	4 (7)
Not useful	0 (0)	0 (0)	0 (0)
Not useful at all	0 (0)	0 (0)	0 (0)
No experience of use	1 (2)	7 (10)	13 (24)
Unknown	0 (0)	1 (1)	0 (0)

ENT, ear, nose, and throat specialists group; GP, general physicians group; IM, internal medicine doctors group; IQR, interquartile range.

RESULTS

Questionnaires were collected from 186 physicians (response rate 36%). Fourteen questionnaires in total were excluded from the analysis; this included 1 ENT, 2 GPs and 5 IMs who left the clinical vignettes blank and 6 IMs who did not examine pollinosis patients during the 2006 cedar pollen season. Overall, responses were received from 172 (32%) physicians consisting of 45 ENTs, 72 GPs and 55 IMs, and were included in the statistical analysis.

BACKGROUND OF SUBJECTS

The ENT and GP groups compared with the IM group demonstrated a trend in seeing a larger number of cedar pollinosis patients each day. Furthermore, approximately 70% of subjects in all groups approved of the validity of the Practical Guideline for the Management of Allergic Rhinitis in Japan (PG-MARJ) (Table 1).¹⁵

REPORTED NUMBER OF PRESCRIBED DRUGS FOR EACH VIGNETTE

The number of drugs reported by physicians that they would prescribe was compared among the 3 groups for each vignette. The drugs that were included in the count were oral drugs, nasal drops, eye drops, co-administered drugs and other required medication. For vignettes 3 to 6, the numbers given

by ENTs were significantly higher than for GPs and IMs ($p < 0.004$) (Fig. 1).

TRENDS IN DRUG PRESCRIPTION FOR EACH TYPE OF DRUG

When comparing the percentage of physicians who reported prescribing second-generation antihistamines using the 95% confidence interval, no differences could be observed among the 3 groups for all vignettes (Fig. 2A).

The percentage of physicians who reported prescribing non-sedative antihistamines was as high as 66% (95% CI: 51%–80%) in the ENT group and 64% (95% CI: 52%–75%) in the GP group, compared with 40% (95% CI: 27%–54%) in the IM group for vignette 2 (data not shown). For all other vignettes, the percentage for non-sedative antihistamines was approximately 25% for all groups.

When the percentage of physicians who reported prescribing nasal corticosteroid drops were compared using a 95% confidence interval, an increasing trend in percentages was found with increasing severity of symptoms in all 3 groups (Fig. 2B). In vignettes 4 to 6, this percentage was clearly higher in the ENT group compared to the other 2 groups.

Comparison of the percentage of physicians who prescribed eye drops using 95% confidence intervals for vignettes 3 to 5 revealed that the percentage in the ENT group was clearly much higher than in the

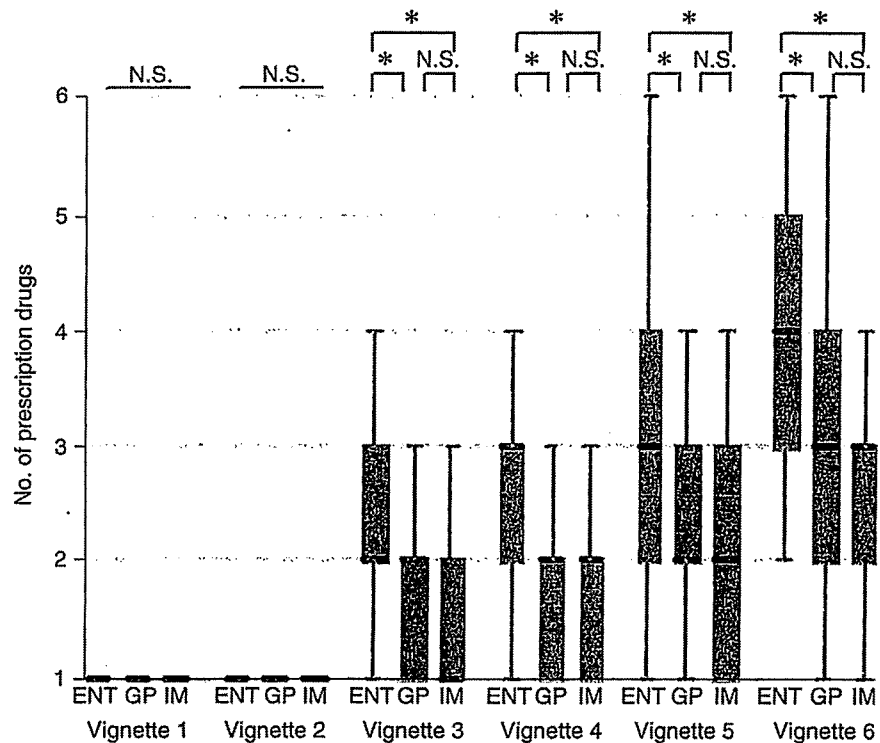


Fig. 1 Box and whisker plots of the number of prescription drugs by ear, nose, and throat specialists (ENTs), general physicians (GPs), and internal medicine doctors (IMs) for each vignette. The vertical bars indicate the range from lower to upper adjacent values. The horizontal boundaries of the boxes represent the first and third quartiles. The thick bars in the boxes indicate medians. NS means, -not statistically significant. *, $p < 0.004$.

other 2 groups (Fig. 2C).

For vignette 6, oral steroids were prescribed by 56% (95% CI: 40%–70%) of the ENT group and 45% (95% CI: 33%–57%) of the GP group, but was lower at 21% in the IM group (95% CI: 11%–34%). For all other vignettes, the percentage for oral steroids prescribed was below 20% in all 3 groups.

As for the percentage of physicians prescribing anti-leukotrienes and vasoconstrictive agents, there were no significant differences among the 3 groups for all vignettes.

POSSIBLE UNDERTREATMENT BY COMPARISON WITH GUIDELINES

In addition, we analyzed the possibility of some prescription patterns to be considered as undertreatment, by referring to the ARIA or PG-MARJ medical practice guidelines.^{1,15} The criteria for classifying rhinitis and assessment of severity differs extensively between the 2 guidelines. The cedar pollen season in Japan lasts for approximately 2 months, and according to the ARIA, most cedar pollinosis patients will be classified as patients with persistent allergic rhinitis. According to the PG-MARJ, however, the severity of

pollinosis is classified into 4 types: mild, moderate, severe, or most severe, on the basis of a patient's nasal symptoms and QOL grading. In the 2 guidelines, it is recommended to use nasal steroids as the first-choice either singularly or concomitantly for persistent moderate/severe allergic rhinitis or moderate/severe/most severe pollinosis. With reference to the above, we analyzed the answers given for vignettes 4 to 6 which represent severe cases and identified those that could potentially be considered as undertreatment.

For vignette 4 which includes severe rhinorrhea and sneezing symptoms, the prescription decided by 33% of physicians in the ENT group, 64% in the GP and 65% in the IM groups could be considered as undertreatment. For vignette 5 which includes severe nasal congestion, possible undertreatment could be identified in 13% of the ENT group, 37% of the GP group and 57% of the IM group. For vignette 6 which involves a case of severe overall symptoms, possible undertreatment could be identified for 4% of the ENT group, 25% of the GP group and 43% of the IM group (Table 2).

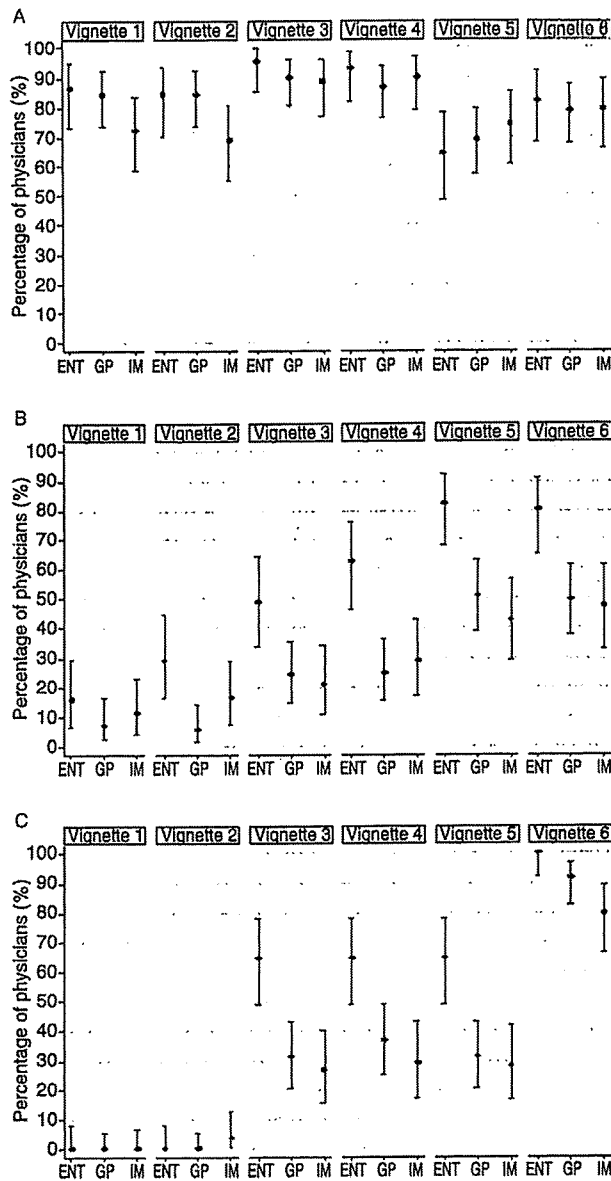


Fig. 2 Percentage of physicians prescribing each type of drug for each vignette in the 3 groups by ear, nose, and throat specialists (ENTs), general physicians (GPs), and internal medicine doctors (IMs). The black circles indicate the simple percentages. The two-tailed bars represent the 95% confidence intervals of the percentages. The one-sided bars represent the 97.5% confidence intervals of the percentages. **A:** Second-generation antihistamines. **B:** Intranasal corticosteroids. **C:** Antiallergic eye drops.

DISCUSSION

In this study we analyzed and compared for the first time the prescription patterns of drugs for cedar pollinosis between physicians classified as ENTs, who are specialists, and GPs and IMs through the use of clinical vignettes.

The research purpose for using clinical vignettes was not to test the knowledge of physicians, but to analyze how the physicians would prescribe medication if they encountered such patients in real life as described by the vignettes. Because responses are likely to be biased in many ways, it is important to analyze the responses given by a strictly defined group of physicians rather than that of individuals.¹³ Recent studies have confirmed the validity of clinical vignettes by comparing them with simulated patient studies. Clinical vignettes are advantageous in that they are more cost effective and practical compared to simulated patients, and most of all, the problem of case-mix that occurs during medical record reviews can be regulated.¹²

In Japan, many cedar pollinosis patients consult otolaryngologists and general physicians. According to past research conducted on the trend of medical consultation for cedar pollinosis patients, 40% consulted otolaryngologists and 30% consulted general physicians, and when questioned about the type of medical institutions visited, 90% of patients consulted clinics.¹⁰ Our questionnaire has confirmed that larger numbers of cedar pollinosis patients consult ENTs and GPs compared to IMs.

Our results suggest that the most obvious differences in the prescription patterns for cedar pollinosis between ENTs, GPs and IMs are those of nasal corticosteroid drops and eye drops. In actuality, many factors including the patients themselves, features of the medical institution and the amount of airborne pollen present in a given season will affect the decision on drug prescription. Therefore, information obtained from our clinical vignettes is insufficient for use by our respondents. It may be that our results here do not represent the trends occurring in the prescription of drugs in actual clinical settings. However, our research does not focus on how well our results reflect true clinical settings, but on discovering any differences in the prescription patterns of ENTs, GPs and IMs for the same given clinical cases. What is important here is the fact that the differences discovered in the prescription patterns among the 3 groups are clearly reflected by the differences in the percentage of physicians prescribing nasal corticosteroid drops and eye drops. In order to improve the quality of cedar pollinosis treatment, it will be extremely necessary to identify the reasons why GPs tend to be reluctant in prescribing nasal corticosteroid drops and eye drops to patients with moderate/severe symptoms. This is intriguing since these GPs in particular examine a stable fraction of patients and understand the effectiveness of the guidelines. The reasons for reluctance may be that they are afraid of systemic side-effects triggered by steroids, or because patients who consult GPs often have complications and are already taking medication.^{8,11} Nevertheless, the clinical vignettes used here were designed so that complica-

Table 2 Potential undertreatment of Japanese cedar pollinosis according to the Allergic Rhinitis and its Impact on Asthma guideline or the Practical Guideline for the Management of Allergic Rhinitis in Japan¹⁵ recommendations

	Vignette 4			Vignette 5			Vignette 6		
	ENT n = 45	GP n = 69	IM n = 52	ENT n = 45	GP n = 71	IM n = 54	ENT n = 45	GP n = 71	IM n = 53
AH	12 (27)	29 (42)	21 (40)	1 (2)	7 (10)	14 (26)	0 (0)	7 (10)	11 (21)
AL	0 (0)	0 (0)	1 (2)	0 (0)	1 (1)	3 (6)	0 (0)	0 (0)	0 (0)
OOt	0 (0)	6 (9)	1 (2)	2 (4)	6 (9)	0 (0)	0 (0)	3 (4)	0 (0)
NsANS	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (4)	0 (0)	0 (0)	0 (0)
ND	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2)	0 (0)	0 (0)	0 (0)
AH + AL	0 (0)	0 (0)	2 (4)	1 (2)	3 (4)	2 (4)	2 (4)	0 (0)	1 (2)
AH + OOt	1 (2)	6 (9)	1 (2)	0 (0)	2 (3)	1 (2)	0 (0)	4 (6)	3 (6)
AH + NsANS	2 (4)	2 (3)	8 (15)	1 (2)	3 (4)	8 (15)	0 (0)	4 (6)	7 (13)
AH + ND	0 (0)	1 (1)	0 (0)	1 (2)	4 (6)	0 (0)	0 (0)	0 (0)	0 (0)
AL + ND	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2)
Total	15 (33)	44 (64)	34 (65)	6 (13)	26 (37)	31 (57)	2 (4)	18 (25)	23 (43)

Number of physicians, (%); ENT, ear, nose, and throat specialists group; GP, general physicians group; IM, internal medicine doctors group; AH, second-generation antihistamines; AL, antileukotriens; OOt, other oral medications (e.g. first-generation antihistamines, herbal medications, etc); NsANS, nonsteroid antiallergic nasal spray; ND, nasal decongestants.

tions would not be included.

Furthermore, when using the medical practice guidelines as a reference, physicians of all 3 groups, including the ENTs who are specialists, may not be fully aware of the severity of symptoms of allergic rhinitis patients. In some cases, drug treatment initiated by these physicians may be insufficient for the control of symptoms. When the prescription patterns suggested by the respondents were compared with the prescriptions recommended by the ARIA guideline and PG-MARJ, a fair proportion of the suggested prescriptions were considered as possible undertreatment. The figures were 25 to 65% of GPs and IMs for the 3 vignettes representing severe cases, and 33% of even the ENT group for the vignette showing a severe case with symptoms of rhinorrhea and sneezing. The response rate of ENTs in this research was high (85%), therefore it will be interesting to know the extent of divergence of the suggested prescription patterns from the guidelines, bearing in mind that these physicians are specialists. Nevertheless, the overall degree of compliance with the guideline was higher for ENTs compared to GPs or IMs with respect to the vignettes representing severe symptoms.

Our study has several limitations. The first is that our research was not conducted during the cedar pollen season. Decisions made by physicians and its patterns may have been elicited more accurately by our vignettes if the research was conducted immediately after the end of the pollen season. Secondly, because our research was based locally in Yamanashi Prefecture, it is somewhat difficult to extend the implications of our results to a nationwide scale. Thirdly, this

research was limited by the low response rate of GPs and IMs, who are non-specialist physicians. The motivation of respondents directly influences the quality of responses when using the clinical vignette method. From this standpoint, although the response rate of the GPs and IMs were 33% and 21%, respectively, it can be suggested that those who kindly responded to this complicated vignette method possessed sufficient motivation. Thus, reliability of the results from these physicians can be considered as high. Furthermore, it may be possible to speculate that GPs/IMs who did not respond, compared to those who did, are consulting the guidelines insufficiently, are prescribing simple medication such as oral medicine and have a higher potential for the undertreatment of patients. If these non-respondent physicians could be included in the statistical analysis, the difference between the prescription patterns found between ENTs and the other 2 groups might become clearer.

To conclude, our investigation of the drug prescription patterns for cedar pollinosis in Yamanashi Prefecture has shown that compared to ENTs, GPs and IMs have a lower tendency to concomitantly prescribe drugs for topical treatment such as nasal corticosteroids and eye drops with oral medication.

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APPENDIX

For the clinical vignettes shown below, please fill out the names of medications that you feel are most appropriate to prescribe.

A: Vignette 1 & 2

The amount of airborne cedar pollen is expected to be normal this season. The patient is a 34-year-old man who has visited with a major complaint of nasal symptoms which started 5 days previously. The visit was made 7 days after the beginning of cedar pollen dispersal. He mentions that he did not experience similar nasal symptoms at this time the previous year. Following medical tests and examinations, he was given a diagnosis of new-onset cedar pollinosis. He has no history of other allergies.

Vignette 1

The occupation of this patient is a clerical worker. The symptoms of rhinorrhea, sneezing and nasal congestion are all mild, and no symptoms involve the eyes.

Vignette 2

The occupation of this patient is a taxi driver. The symptoms of rhinorrhea, sneezing and nasal congestion are all mild, and no symptoms involve the eyes.

B: Vignettes 3-6

The amount of airborne cedar pollen is expected to be normal this season. The patient is a 34-year-old man who has visited with a major complaint of nasal symptoms which started 7 days previously. The visit was made 10 days after the beginning of cedar pollen dispersal. The level of airborne cedar pollen is estimated to reach its peak 1 week after his visit. He had been experiencing similar nasal symptoms around this time for the past several years, and apparently had medication prescribed by other clinics, although details are unknown. Following medical tests and examinations, he was given a diagnosis of cedar pollinosis. He has no history of other allergies.

Vignette 3

The occupation of this patient is a clerical worker. The symptoms of rhinorrhea, sneezing and nasal congestion are moderate, and symptoms involving the eyes are mild.

Vignette 4

The occupation of this patient is a clerical worker. The symptoms of rhinorrhea and sneezing are severe, but nasal congestion is mild. Symptoms involving the eyes are mild.

Vignette 5

The occupation of this patient is a clerical worker. The symptoms of rhinorrhea and sneezing are mild, but nasal congestion is severe. Symptoms involving the eyes are mild.

Vignette 6

The occupation of this patient is a clerical worker. The symptoms of rhinorrhea, sneezing and nasal congestion are all severe. Symptoms involving the eyes are also severe.

REFERENCES

1. Bousquet J, Van Cauwenberge P, Khaltaev N. Allergic rhinitis and its impact on asthma. *J Allergy Clin Immunol* 2001;108:S147-334.
2. Okuda M. Epidemiology of Japanese cedar pollinosis throughout Japan. *Ann Allergy Asthma Immunol* 2003;91:288-96.
3. Kaneko Y, Motohashi Y, Nakamura H, Endo T, Eboshida A. Increasing prevalence of Japanese cedar pollinosis: a meta-regression analysis. *Int Arch Allergy Immunol* 2005;136:365-71.
4. Fukuhara S, Nishimura M, Nordyke RJ, Zaher CA, Peabody JW. Patterns of care for COPD by Japanese physicians. *Respirology* 2005;10:341-8.
5. Bratton SL, Odetola FO, McCollegan J, Cabana MD, Levy FH, Keenan HT. Regional variation in ICU care for pediatric patients with asthma. *J Pediatr* 2005;147:355-61.
6. Katz SJ, Lantz PM, Janz NK *et al.* Surgeon perspectives about local therapy for breast carcinoma. *Cancer* 2005;104:1854-61.
7. Greenfield S, Kaplan SH, Kahn R, Ninomiya J, Griffith JL. Profiling care provided by different groups of physicians: effects of patient case-mix (bias) and physician-level clustering on quality assessment results. *Ann Intern Med* 2002;136:111-21.
8. Fokkens WJ. Nasal corticosteroids, first choice in moderate to severe allergic rhinitis. What prevents general practitioners from using them? *Allergy* 2003;58:724-6.
9. Demoly P, Allaert FA, Lecasble M. ERASM, a pharmacoepidemiologic survey on management of intermittent allergic rhinitis in every day general medical practice in France. *Allergy* 2002;57:546-54.
10. Okuda M, Ohkubo K, Goto M. [Are otolaryngologists specialist for pollinosis?]. *Averugi* 2004;53:1144-51 (in Japanese).
11. Van Hoescke H, Vastesaeager N, Dewulf L, Sys L, van Cauwenberge P. Classification and management of allergic rhinitis patients in general practice during pollen season. *Allergy* 2006;61:705-11.
12. Peabody JW, Luck J, Glassman P *et al.* Measuring the quality of physician practice by using clinical vignettes: a prospective validation study. *Ann Intern Med* 2004;141:771-80.
13. Veloski J, Tai S, Evans AS, Nash DB. Clinical vignette-based surveys: a tool for assessing physician practice variation. *Am J Med Qual* 2005;20:151-7.
14. Ministry of Health, Labour and Welfare. [Survey of medical doctors, dentists, and pharmacists. The number of medical doctors sorted by their settings in Yamanashi Prefecture]. 2004. Available from: http://www.dbtk.mhlw.go.jp/toukei/data/180/2004/toukeihyou/0005030/t0110373/E50019_001.html (in Japanese). Cited 2007 September 16.
15. Okuda M, Baba K, Konno A *et al.* [Practical Guideline for the Management of Allergic Rhinitis in Japan], 5th edn. Tokyo: Life Science, 2005 (in Japanese).

Analysis of T-helper responses and FOXP3 gene expression in patients with Japanese cedar pollinosis

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ABSTRACT

Background: Evidence has been accumulated indicating that regulatory T (T-reg) cells play a crucial role in the maintenance of peripheral T-cell tolerance to allergens. To explore the role of FOXP3, which is required for the development of T-reg cells, in allergen-specific immune responses, we examined the relationship between the alteration of FOXP3 gene expression and in vitro immune responses against allergens.

Methods: Peripheral blood mononuclear cells obtained from 19 human histocompatibility leukocyte antigens (HLA)-DPB1*0501 donors, including patients with Japanese cedar pollinosis and nonallergic healthy donors, were stimulated with Cry j 1 p61–75 peptide. On day 7, T cells were tested for peptide-specific reactivity in IFN- γ and interleukin (IL)-5 enzyme-linked immunospot (ELISPOT) assays. Real-time quantitative RT-PCR was performed to assess relative change of FOXP3 gene expression before and after in vitro stimulation. Neutralization assays using anti-glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) and anti-IL-10 monoclonal antibody were also performed.

Results: Of 14 patients with allergic pollinosis tested, 10 responders displayed T-helper type 2 (Th2)-polarized reactivity to Cry j 1 p61–75, and 2 donors showed Th0 responses. Notably, the change of FOXP3 gene expression in donors showing peptide-specific T-helper responses was significantly lower than that in nonresponders, regardless of allergic pollinosis.

Conclusion: Our data indicate that FOXP3 is functional in nonallergic healthy donors as well as allergic patients, and FOXP3-expressing T cells may be responsible for the down-regulation of allergen-specific T-helper responses in individuals. A better understanding of the nature and specificity of FOXP3-expressing T cells in a suppressive mechanism is necessary to develop new immunotherapies against allergic rhinitis.

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Key words: Allergic rhinitis, Cry j 1, FOXP3, immunotherapy, Japanese cedar pollinosis, regulatory T cells, Th2, suppressive

Allergic diseases, including rhinitis, are characterized by the presence of specific IgEs to allergens such as pollens, house-dust mites, and animal proteins. T helper 2 (Th2) cells and an associated cytokine secretion profile, including interleukin (IL)-4, IL-5, and IL-13, are thought to play a pivotal role in the initiation and perpetuation of allergic diseases. For example, switching B cells to IgE production and the accumulation of eosinophils are under the control of Th2 cells via IL-4 and IL-5, respectively.

A subset of regulatory T (T-reg) cells has been recognized as an immunosuppressive T-cell subset capable of the induction and maintenance of immunologic tolerance to self and nonself antigens.^{1–3} Currently, two different categories of T-reg cells, naturally occurring CD4⁺CD25⁺ T-reg cells and antigen-induced T-reg cells, have been reported.^{4,5} Evidence has been accumulated indicating that these T-reg cells play a crucial role in the prevention of inappropriate Th2 responses

in allergic diseases such as asthma, rhinitis, and dermatitis.^{6,7} Naturally occurring CD4⁺CD25⁺ T-reg cells are able to inhibit proliferation and cytokine production by effector T cells in an antigen-nonspecific, cytokine-independent, but cell-cell contact-dependent manner. Ling *et al.* reported that allergen-specific Th2 cells from allergic patients were significantly enhanced by the depletion of CD4⁺CD25⁺ T cells.⁸ Bellinghausen *et al.* also indicated that CD4⁺CD25⁺ T cells from both nonatopic donors and atopic patients inhibited the proliferation and Th2 cytokine production of CD4⁺CD25⁻ T cells.⁹ On the other hand, antigen-induced T-reg cells are induced in the presence of IL-10, produce high levels of IL-10, and suppress the proliferation and cytokine production of effector T cells in an IL-10-dependent manner. Akdis *et al.* showed that IL-10-producing allergen-specific CD4⁺ T cells represented the predominant subset with significantly high frequency in comparison with IL-4- and IFN- γ -producing T cells in healthy individuals.¹⁰

Recently, it has been shown that FOXP3, which encodes a forkhead/winged-helix transcription repressor termed Scrf, can be specifically expressed in CD4⁺CD25⁺ T-reg cells.^{11,12} Actually, a mutation in the gene encoding FOXP3 results in a disease called immune dysregulation, polyendocrinopathy, and enteropathy, X-linked (IPEX) syndrome. Patients with IPEX have a variety of allergic symptoms including food allergies, increased serum IgE levels, and severe eczema.¹³ Alternatively, FOXP3-transduced CD4⁺ naïve T cells exerted suppression in a cell-cell contact-dependent manner similar to CD4⁺CD25⁺ T-reg cells.¹⁴ Thus, the FOXP3

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gene is a crucial regulatory gene for the development and function of T-reg cells. To explore the role of the FOXP3 gene in allergen-specific immune responses, in particular Th2 response, we examined the relationship between the alteration of FOXP3 gene expression and *in vitro* immune responses against allergens.

In this study, subjects with Japanese cedar pollinosis were the target population. Japanese cedar pollinosis caused by exposure to *Cryptomeria japonica* pollen is one of the most prevalent allergic diseases in Japan, and two major allergens, Cryj1 and Cryj2, have been isolated and specific IgE is detected in patients suffering from pollinosis. To date, a number of human histocompatibility leukocyte antigens (HLA) class II-restricted Cryj1 and Cryj2 epitopes defined by CD4⁺ T cell have been identified.¹⁵ Among these epitopes, we used an HLA-DPB1*0501-restricted Cryj1 p61-75 peptide for the induction of allergen-specific T-cell responses. Our findings provide insights into the further elucidation of the mechanism of suppression by T-reg cells as well as the development of novel therapies for allergic diseases.

MATERIALS AND METHODS

Subjects

Peripheral blood was obtained from 5 nonallergic healthy donors and 14 allergic patients suffering from Japanese cedar pollinosis, a diagnosis made based on their case history, clinical symptoms, and the presence of allergen-specific IgE in the sera (radioallergosorbent test class, >2). The study was approved by the Institutional Review Board at Gunma University Hospital. Written informed consent was obtained from each individual. Subject characteristics are summarized in Table 1. Heparinized venous blood (40 mL) was obtained from all subjects, and peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over Ficoll-Hypaque gradients (Amersham Biosciences, Uppsala, Sweden), washed, and counted after staining with trypan blue dye. HLA-DPB1*0501 genotyping was performed using a commercial DP5 typing panel of PCR primers according to the manufacturer's instructions (Dýnal, Oslo, Norway).

Cell Line and Peptide

The H0301 cell line, HLA-DPB1*0501 homozygous Epstein-Barr virus-transformed B lymphocytes, used as the peptide-presenting cells in enzyme-linked immunospot (ELISPOT) assays, was kindly provided by Dr. Nishimura (Kumamoto University, Kumamoto, Japan). It was maintained in RPMI-1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/mL of penicillin, 100 µg/mL of streptomycin, 2 mM of L-glutamine (all reagents from Invitrogen, Grand Island, NY). HLA-DPB1*0501-restricted Cryj1 p61-75 peptide (GATRDRPLWIFSGN) was synthesized using standard N-(9-fluorenyl) methoxycarbonyl methodology. The peptide was stored as a lyophilized preparation. The amino acid sequence was confirmed by mass spectrometric analysis.¹⁵

IFN-γ and IL-5 ELISPOT Assays for Cryj1 Peptide-Specific T-Cell Responses

T-helper responses to Cryj1 peptide were evaluated by both IFN-γ (Th1 type) and IL-5 (Th2 type) ELISPOT assays.

Table 1 Clinical characteristics of human histocompatibility leukocyte antigen-DPB1*0501⁺ donors in this study

	Age/Sex	Japanese Cedar Pollen-Specific IgE (class) (UA/mL)
Pollen allergic		
AR-1	42 M	1.30 (2)
AR-2	32 M	3.67 (3)
AR-3	34 M	16.5 (3)
AR-4	25 F	6.15 (3)
AR-5	23 F	0.85 (2)
AR-6	33 F	10.7 (3)
AR-7	36 M	3.85 (3)
AR-8	33 M	1.39 (2)
AR-9	33 M	2.09 (2)
AR-10	52 F	0.80 (2)
AR-11	49 F	53.5 (5)
AR-12	32 F	>100 (6)
AR-13	24 F	31.7 (4)
AR-14	48 F	13.1 (3)
Healthy control		
HD-1	28 F	<0.34 (0)
HD-2	29 F	<0.34 (0)
HD-3	33 M	<0.34 (0)
HD-4	27 F	<0.34 (0)
HD-5	55 F	<0.34 (0)

AR = allergic rhinitis; HD = healthy donor; F = females; M = males.

PBMC (4×10^6) were cultured in the presence of Cryj1 peptide (10 µg/mL) in wells of 24-well tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) in a final volume of 2 mL of AIM-V medium (Invitrogen, Grand Island, NY) containing 10% (v/v) human AB serum. After 7 days of incubation at 37°C, PBMCs were harvested, washed and tested in ELISPOT assay. ELISPOT assays were performed in 96-well flat-bottom plates with nitrocellulose membrane inserts (Millipore, Bedford, MA) as previously described.¹⁶ Briefly, the plates were coated overnight at 4°C with 10 µg/mL of anti-human IFN-γ monoclonal antibody (monoclonal antibody [mAb]; 1-D1K; Mabtech, Nacka Strand, Sweden), or 5 µg/mL of anti-human IL-5 mAb (TRFK5; BD Pharmingen, San Diego, CA) in PBS. H0301 cells used as antigen-presenting cells were pulsed with a Cryj1 p61-75 peptide (10 µg/mL) and plated in triplicate wells at 1×10^5 cells/well. The responder cells (1×10^5 cells/well) were added to AIM-V medium at a final volume of 200 µL. PBMCs stimulated with 12.5 ng/mL of phorbol 12-myristate 13-acetate and 1 µg/mL of ionomycin were used as a positive control. The plates were incubated at 37°C for 20 hours for IFN-γ assessment and 40 hours for IL-5 assessment. After incubation, the plates were washed with PBS/0.05% Tween 20, and supplemented with biotinylated anti-IFN-γ mAb (7-B6-1; Mabtech) or biotinyl-

ated anti-IL-5 mAb (JES1-5A10; BD Pharmingen). After 2 hours incubation, plates were washed with PBS/0.05% Tween 20 and developed with the avidin-peroxidase complex (Vectastain Elite kit; Vector, Burlingame, CA) for 1 hour. The 3-amino-9-ethyl-carbozole (Sigma, St. Louis, MO) was added and incubated for 5 minutes for the IFN- γ ELISPOT assay and the TMB (3,3',5,5'-tetramethylbenzidine; Vector Laboratories) substrate for peroxidase was added and incubated for 10 minutes for the IL-5 ELISPOT assay. The mean number of spots in control wells (no peptide) was subtracted from the mean number of spots in experimental wells. A T-cell response to a given Cry j 1 peptide was considered to be positive if at least 10 cells per 1×10^5 responder cells secreted IFN- γ or IL-5, as described by Nagorsen *et al.*¹⁷

Real-Time Quantitative RT-PCR

To assess relative FOXP3 gene expression, real-time quantitative RT-PCR was performed. RNA was extracted from isolated PMBCs and 7-day cultured responder cells using the RNeasy mini kit (Qiagen, Valencia, CA). Quantitative RT-PCR was performed using the FullVelocity SYBR Green QRT-PCR Master Mix in a total volume of 25 μ L and the FullVelocity cycling PCR program on an MX 3000P (Stratagene, La Jolla, CA). The melting curve was recorded at the end of every run to assess product specificity. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as an internal control gene. PCR primers used in this study were as follows: FOXP3 forward primer, 5'-GTTACACGCATGTTTGCCTTC-3'; reverse primer, 5'-GCACAAAGCACTTGTGCAGACTC-3'; GAPDH forward primer, 5'-GCACCGTCAAGGCTGAGAAC-3'; reverse primer, 5'-ATGGTGGTGAAGACGCCAGT-3'. The relative changes in FOXP3 gene expression were determined by the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = (Ct_{FOXP3} - Ct_{GAPDH})_{day 7} - (Ct_{FOXP3} - Ct_{GAPDH})_{day 0}$.

Neutralization Assays

Isolated PMBCs were stimulated with Cry j 1 p61-75 (10 μ g/mL) in the presence of anti-IL-10 mAb, anti-GITR mAb, or control IgG (R&D Systems, Minneapolis, MN) at a final concentration of 10 μ g/mL (as suggested by the supplier). Cells were cultured under the same conditions as described previously in "IFN- γ and IL-5 ELISPOT assays for Cry j 1 peptide-specific T-cell responses." After 7 days, cells were harvested and tested for cytokine production in ELISPOT assay.

Statistical Analysis

Mann-Whitney's *U* test was used for statistical analysis of data. The values of $p < 0.05$ were considered significant. Analyses were performed using Statcel2 (OMS Publishing, Tokorozawa, Japan).

RESULTS

T-Helper Responses to Cry j 1 p61-75 of PMBCs Obtained from HLA-DPB1*0501 Donors after *In Vitro* Stimulation

A Cry j 1 p61-75 was tested in *in vitro* stimulation (IVS) for the ability to induce Cry j 1-specific T-helper responses in 19 HLA-DPB1*0501 donors (14 patients with Japanese cedar pol-

Table 2 Analysis of T-cell responses to Cry j 1 p61-75 in human histocompatibility leukocyte antigens-DPB1*0501⁺ donors

	IFN- γ	IL-5
Pollen allergic		
AR-1	0	16
AR-2	6	1
AR-3	0	35
AR-4	0	29
AR-5	0	13
AR-6	0	28
AR-7	0	0
AR-8	17	16
AR-9	10	14
AR-10	2	27
AR-11	0	21
AR-12	0	35
AR-13	0	31
AR-14	7	36
Healthy control		
HD-1	6	0
HD-2	16	17
HD-3	8	0
HD-4	26	0
HD-5	0	1

*Data are the mean number of spots/ 10^5 T cells/well in enzyme-linked immunospot assays.

Bold number indicates positive for T-helper responses to Cry j 1 peptide.

AR = allergic rhinitis; HD = healthy donor; IFN- γ = interferon γ ; IL = interleukin.

linosis and 5 nonallergic healthy donors). PMBCs obtained from these donors were cocultured in the presence of this peptide. After 7-day IVS (1xIVS), T cells were evaluated for peptide-specific reactivity in IFN- γ (Th1 type) and IL-5 (Th2 type) ELISPOT assays. As expected, 12 of 14 pollen-allergic donors tested showed T-helper responses to Cry j 1 p61-75 (Table 2). Of these, 10 of 12 responders displayed Th2-polarized reactivity to Cry j 1 peptide, and the remaining 2 donors showed Th0 responses. On the other hand, 2 of 5 nonallergic healthy donors showed Th0 and Th1 response each, and no response was detected in three donors. Next, we examined the possibility that Th2 responses to Cry j 1 peptide correlated with the specific IgE level. Twelve patients showing significant IL-5 production were evaluated. As shown in Fig. 1, there was a significant correlation between the number of IL-5 spots and specific IgE level ($r = 0.70$; $p = 0.02$).

Alteration of FOXP3 Gene Expression and T-Helper Responses

First, we compared the relative expression of FOXP3 gene in PMBCs between pollen-allergic patients and nonallergic healthy donors; however, there was no significant difference (data not shown). Next, FOXP3 gene expression by T cells

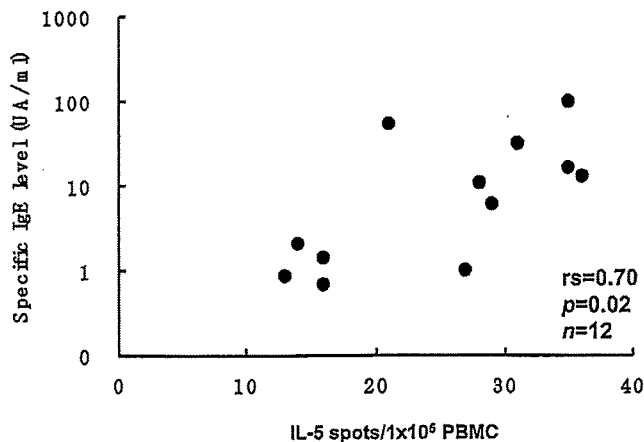


Figure 1. Correlations of T-helper 2 (Th2) responses to Cry j 1 p61-75 with specific IgE level. Peripheral blood mononuclear cells obtained from HLA-DPB1*0501 donors were cocultured in the presence of Cry j 1 p61-75 peptide. After 7-day in vitro stimulation (IVS; 1xIVS), T cells were evaluated for peptide-specific reactivity in IL-5 ELISPOT assays.

before and after IVS was examined, and the relative change in FOXP3 gene expression varied. The relative change in FOXP3 gene expression and the type of T-helper response are summarized in Table 3. Nine of 19 donors showed increased expression of FOXP3, but 10 did not. We next analyzed the relationship between the change of FOXP3 gene expression and several parameters. As shown in Fig. 2, there was no significant difference in the relative change of FOXP3 gene expression between pollen-allergic patients and nonallergic healthy donors. Interestingly, the change in FOXP3 gene expression in donors showing T-helper responses, including Th0, Th1, or Th2, was significantly lower than that in nonresponders ($p < 0.01$). Moreover, there was no significant difference between donors with Th0 and Th2 responses in the changes of FOXP3 gene expression; however, donors with Th0 and Th2 responses showed a smaller change of FOXP3 gene expression compared with those with no response (Th0 versus no response, $p < 0.05$; Th2 versus no response, $p < 0.01$). These results suggested that the FOXP3 gene may affect T-helper responses regardless of the type of responses.

Suppressor Mechanisms of Regulatory Function in T-helper Responses

To investigate the suppression mechanisms in nonresponders, including both pollen-allergic patients and nonallergic healthy donors, a neutralization assay was performed. The addition of anti-IL-10 and anti-glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) to a coculture of PBMCs with Cry j 1 peptide affected T-helper responses, as shown in Fig. 3. The addition of anti-GITR to cocultures of PBMCs with Cry j 1 p61-75 peptide in pollen-allergic patients could induce Th2 responses, whereas that in nonallergic healthy donors it induced Th1 responses. Interestingly, two pollen-allergic patients, AR-2 and AR-7, showed different types of T-helper response by adding anti-IL-10, *viz.*, patient AR-2 and AR-7 revealed enhanced Th2 and Th1 responses, respectively.

Table 3 Summary of T-cell responses to Cry j 1 p61-75 and alteration of mRNA expression of FOXP3 in human histocompatibility leukocyte antigens-DPB1*0501⁺ donors

	T-Helper Type*	Relative Change in FOXP3 Gene Expression
Pollen allergic		
AR-1	Th2	0.34
AR-2	NR	2.43
AR-3	Th2	0.61
AR-4	Th2	1.35
AR-5	Th2	0.80
AR-6	Th2	1.87
AR-7	NR	2.08
AR-8	Th0	0.52
AR-9	Th0	0.93
AR-10	Th2	0.44
AR-11	Th2	1.39
AR-12	Th2	1.41
AR-13	Th2	0.51
AR-14	Th2	0.45
Healthy control		
HD-1	NR	1.92
HD-2	Th0	0.38
HD-3	NR	2.07
HD-4	Th1	0.36
HD-5	NR	2.35

*Th0, Th1, or Th2 assignment for peptide reactivity reflects donor responses of ≥ 10 spots/ 10^5 T cells as determined in interferon γ (IFN- γ) or interleukin (IL)-5 ELISPOT assays, respectively.

AR = allergic rhinitis; HD = healthy donor; NR = no response; Th0 = type 0 response; Th1 = type 1 response; Th2 = type 2 response.

DISCUSSION

In this study, we investigated the role of FOXP3 gene in the suppression mechanism of Th responses, in particular, the Th2 response to Cry j 1-derived peptide. First, we have shown the induction of T-helper responses in Japanese cedar pollinosis using an HLA-DPB1*0501-restricted Cry j 1 p61-75 peptide. To date, a number of Cry j 1-derived epitopes defined by various HLA class II-restricted, CD4⁺ T cells have been identified.¹⁵ In general, it is accepted that PBMCs of allergic patients preferentially induced Th2 rather than Th1 responses under stimulation with certain allergens; however, proteins or longer peptides containing multiple epitopes may induce various T-helper responses, and the extent of T-helper responses to each epitope may also vary. To simplify the evaluation of T-helper responses, we selected HLA-DPB1*0501-restricted Cry j 1 p61-75 as an allergen for this experiments. Hori *et al.* found that the frequency of HLA-DP5 was significantly increased in patients with Japanese cedar

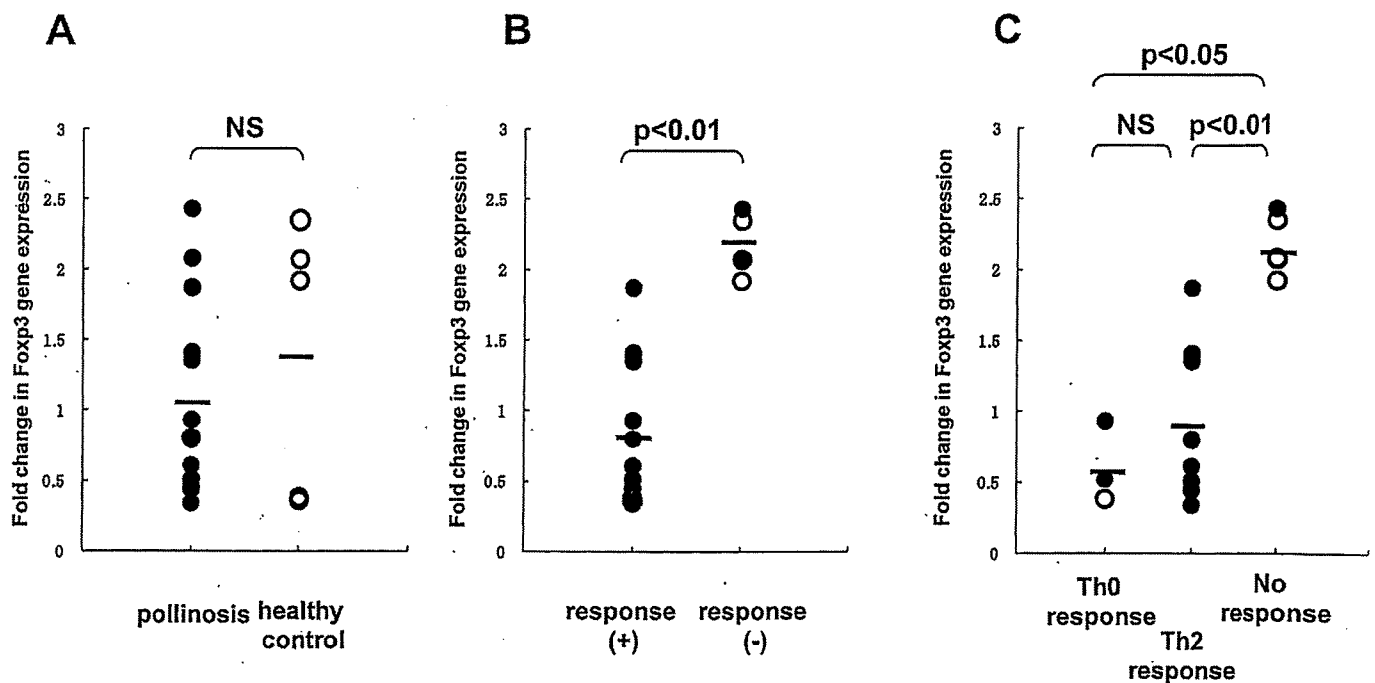


Figure 2. Correlation of alteration of FOXP3 gene expression with T-helper responses. Fold change in FOXP3 gene expression before and after *in vitro* stimulation (IVS) was plotted against (A) pollinosis versus healthy control, (B) positive and negative responsiveness to Cry j 1 p61–75, (C) T-helper 0 (Th0; IFN- γ /IL-5)-type, Th2 (IL-5)-type, and no responses to Cry j 1 p61–75 (closed circles, pollinosis donors; open circles, healthy controls). Th0, Th1, or Th2 assignment for peptide reactivity reflects donor responses of ≥ 10 spots/ 10^5 T cells as determined in IFN- γ or IL-5 ELISPOT assays, respectively. The change of FOXP3 gene expression in donors showing T-helper responses, including Th0 (IFN- γ /IL-5), Th1 (IFN- γ), or Th2 (IL-5) type, was significantly lower than that in nonresponders ($p < 0.01$). Moreover, donors with Th0 and Th2 responses showed lower change of FOXP3 gene expression compared to those with no response, respectively (Th0 versus no response, $p < 0.05$; Th2 versus no response, $p < 0.01$).

pollinosis.¹⁸ Moreover, Sone *et al.* reported that a combination of Cry j 1 p61–75 and DPB1*0501 tended to induce Th2-like cells in T-cell clones.¹⁹ As expected, PBMCs from most allergic patients examined produced IL-5 under stimulation with Cry j 1 p61–75. Meanwhile, those from nonallergic healthy donors did not show the Th2 response. Interestingly, there was a significant correlation between IL-5 production and the specific IgE level. Kimura *et al.* have reported that the production of Th2 cytokines, including IL-4 and IL-13, by PBMCs on stimulation with house-dust mites showed a close positive correlation with house-dust mite IgE radioallergosorbent test.²⁰ Macaubas *et al.* have also shown that the production of IgE antibodies against major house-dust mite allergen Der p 1 was significantly associated with IL-4 and IL-9 mRNA responses and with IL-5 and IL-13 protein responses against crude house-dust mites.²¹ Thus, our findings confirmed that allergen-specific Th2 responses were likely to be the most important in the up-regulation of *in vivo* IgE synthesis in allergic patients.

In the past 10 years, immunologic research regarding T-reg cells has progressed rapidly and has become extremely popular.^{1–5} FOXP3 is considered to be the most specific and reliable marker of T-reg cells.^{11,12} In our study, when stimulated with Cry j 1 p61–75, the FOXP3 gene was up-regulated in donors with undetectable T-helper response. Although FOXP3 is preferentially and stably expressed in naturally occurring T-reg cells, it is controversial whether FOXP3 is

expressed by induced CD4⁺CD25⁺ T-reg cells. Walker *et al.* reported that FOXP3-expressing CD4⁺CD25⁺ T-reg cells were generated by plate-bound anti-CD3/soluble anti-CD28 stimulation,²² whereas Yagi *et al.* have shown that T cell receptor stimulation of CD4⁺CD25⁻ naïve T cells failed to elicit FOXP3 expression at the gene or protein level.¹⁴ In addition, it remains unknown whether Cry j 1-specific T-reg cells exist in the periphery in nonallergic healthy donors and/or patients with Japanese cedar pollinosis; therefore, it is unclear which type of T-reg cells are more related to the suppression of Cry j 1-specific T-helper responses; however, our results suggest that the participation of the FOXP3 gene could be required to suppress allergen-specific T-helper responses. Moreover, our results showed no difference between nonallergic healthy donors and patients with Japanese cedar pollinosis in the relative expression of FOXP3 gene in PBMCs, suggesting that FOXP3 function in patients may not be impaired compared with nonallergic healthy donors.

Neutralization assays revealed that two suppressive mechanisms may affect the suppression of Cry j 1-specific T-helper response. GITR is also constitutively expressed by naturally occurring CD4⁺CD25⁺ T-reg cells and cross-linking of GITR by a specific antibody has been shown to abrogate CD4⁺CD25⁺ T-cell-mediated suppression *in vitro*.²³ In our study, two nonresponsive patients to Cry j 1 p61–75 showed increased Th2 response by adding anti-GITR, whereas three nonallergic healthy donors showed increased Th1 response

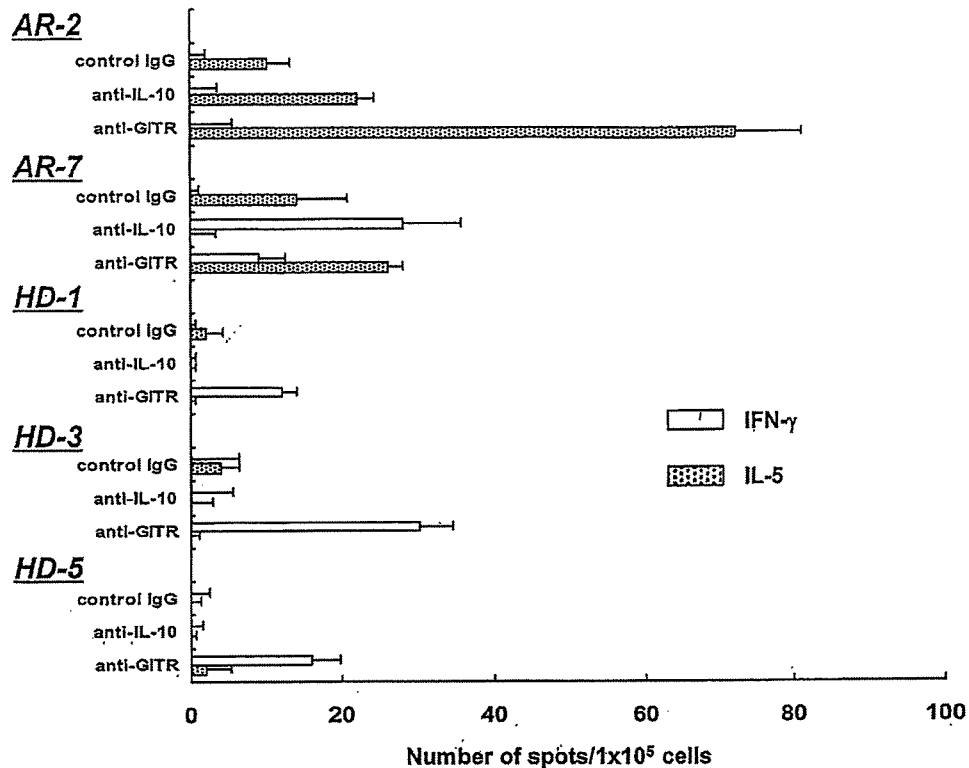


Figure 3. Suppressor mechanisms in donors with no response to Cry j 1 p61–75. Peripheral blood mononuclear cells (PBMCs) were cocultured with Cry j 1 p61–75 peptide in the presence of anti-IL-10 and anti-GITR. T cells were evaluated for peptide-specific reactivity in IFN- γ and IL-5 ELISPOT assays on day 7. The addition of anti-IL-10 and anti-GITR enhanced T-helper responses, including Th1 and/or Th2 responses. Data are expressed as the mean number of spots \pm SD of triplicate cultures.

rather than Th2 response, suggesting that naturally occurring CD4⁺CD25⁺ T-reg cells were able to inhibit both Cry j 1-specific Th1 and Th2 responses. Bellinghausen *et al.* have also shown that CD4⁺CD25⁺ T-reg cells in atopic patients with allergic rhinitis are able to inhibit Th1 as well as Th2 cytokine production.⁹ Moreover, Cosmi *et al.* have reported that Th2 cells were less sensitive than Th1 cells to regulation by CD4⁺CD25⁺ T-reg cells.²⁴ On the other hand, neutralization of IL-10 was also able to induce Cry j 1-specific T-helper responses in patient AR-7 and AR-2. In the experiment shown here, IL-10 ELISPOT assays were also performed under stimulation with Cry j 1 p61–75; however, significant IL-10 production was not detected in our system (data not shown). Minang *et al.* have reported that allergen-induced IL-10 down-regulates Th1- but not Th2-type responses.²⁵ In contrast, Akdis *et al.* have revealed that IL-10-producing allergen-specific CD4⁺ T cells suppressed allergen-specific Th2 cells.¹⁰ Currently, not only the nature of different T-reg cell populations, but also the relationship between naturally occurring CD4⁺CD25⁺ T-reg cells and antigen-induced T-reg cells and the different susceptibility of Th1 and Th2 cells to T-reg cells remain controversial. In addition, other factors such as HLA class II allele, the allergic status of the donor, and type of antigens and their concentration might also influence suppressive mechanisms in individuals.^{26,27} Thus, suppression of allergen-specific T-helper responses appears to be regulated by complex interaction between various factors. Additional

investigation is required to elucidate the exact suppressive mechanism.

Taken together, our data indicate that FOXP3 is functional in nonallergic healthy donors as well as allergic patients, and FOXP3-expressing T cells may be, in part, responsible for the down-regulation of allergen-specific T-helper responses in individuals; therefore, the induction of FOXP3-expressing T-reg cells appears crucial for the maintenance of immunosuppression or the effectiveness of allergen immunotherapy. A better understanding of the nature and specificity of FOXP3-expressing T cells in the suppressive mechanism is necessary to design better and develop more effective immunotherapy in the future.

REFERENCES

1. Levings MK, Sangregorio R, and Roncarolo MG. Human CD25⁺CD4⁺ T regulatory cells suppress naïve and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med* 193:1295–1302, 2001.
2. Ng WR, Duggan PJ, Ponchel F, et al. Human CD4⁺CD25⁺ cells: A naturally occurring population of regulatory T cells. *Blood* 98:2736–2744, 2001.
3. Jonuleit H, Schmitt E, Stassen M, et al. Identification and functional characterization of human CD4⁺CD25⁺ T cell with regulatory properties isolated from peripheral blood. *J Exp Med* 193:1285–1294, 2001.
4. Hawrylowicz CM. Regulatory T cells and IL-10 in allergic inflammation. *J Exp Med* 202:1459–1463, 2005.

5. Taylor A, Verhagen J, Akdis CA, et al. T regulatory cells and allergy. *Microbes Infect* 7:1049–1055, 2005.
6. Robinson DS, Larche M, and Durham SR. Tregs and allergic disease. *J Clin Invest* 114:1389–1397, 2004.
7. Akdis M, Blaser K, and Akdis CA. T regulatory cells in allergy: Novel concepts in the pathogenesis, prevention, and treatment of allergic diseases. *J Allergy Clin Immunol* 116: 961–968, 2005.
8. Ling EM, Smith T, Nguyen XD, et al. Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet* 363:608–615, 2004.
9. Bellinghausen I, Klostermann B, Knop J, et al. Human CD4+CD25+ T cells derived from the majority of atopic donors are able to suppress TH1 and TH2 cytokine production. *J Allergy Clin Immunol* 111:862–868, 2003.
10. Akdis M, Verhagen J, Taylor A, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med* 199:1567–1575, 2004.
11. Hori S, Nomura T, and Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057–1061, 2003.
12. Kim JM, and Rudensky A. The role of the transcription factor Foxp3 in the development of regulatory T cells. *Immunol Rev* 212:86–98, 2006.
13. Bennett CL, Christie J, Ramsdell F, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 27:20–21, 2001.
14. Yagi H, Nomura T, Nakamura K, et al. Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. *Int Immunol* 16:1643–1656, 2004.
15. Sone T, Morikubo K, Miyahara M, et al. T cell epitopes in Japanese cedar (*Cryptomeria japonica*) pollen allergens: Choice of major T cell epitopes in Cry j 1 and Cry j 2 toward design of the peptide-based immunotherapeutics for the management of Japanese cedar pollinosis. *J Immunol* 161: 448–457, 1998.
16. Sakakura K, Chikamatsu K, Furuya N, et al. Toward the development of multi-epitope p53 cancer vaccines: An in vitro assessment of CD8+ T-cell responses to HLA class I-restricted wild-type sequence p53 peptides. *Clin Immunol* 125:43–51, 2007.
17. Nagorsen D, Keilholz U, Rivoltini L, et al. Natural T-cell response against MHC class I epitopes of epithelial cell adhesion molecule, her-2/neu, and carcinoembryonic antigen in patients with colorectal cancer. *Cancer Res* 60:4850–4854, 2000.
18. Hori T, Kamikawaji N, Kimura A, et al. Japanese cedar pollinosis and HLA-DP5. *Tissue Antigens* 47:485–491, 1996.
19. Sone T, Morikubo K, Shimizu K, et al. Peptide specificity, HLA class II restriction, and T-cell subsets of the T-cell clones specific to either Cry j 1 or Cry j 2, the major allergens of Japanese cedar (*Cryptomeria japonica*) pollen. *Int Arch Allergy Immunol* 119:185–196, 1999.
20. Kimura M, Tsuruta S, and Yoshida T. IL-4 production by PBMCs on stimulation with mite allergen is correlated with the level of serum IgE antibody against mite in children with bronchial asthma. *J Allergy Clin Immunol* 105:327–332, 2000.
21. Macaubas C, Sly PD, Burton P, et al. Regulation of T-helper cell responses to inhaled allergen during early childhood. *Clin Exp Allergy* 29:1223–1231, 1999.
22. Walker MR, Kasprovicz DJ, Gersuk VH, et al. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. *J Clin Invest* 112:1437–1443, 2003.
23. Shimizu J, Yamazaki S, Takahashi T, et al. Stimulation of CD25+CD4+ regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* 3:135–142, 2002.
24. Cosmi L, Liotta F, Angeli R, et al. Th2 cells are less susceptible than Th1 cells to the suppressive activity of CD25+ regulatory thymocytes because of their responsiveness to different cytokines. *Blood* 103:3117–3121, 2004.
25. Minang JT, Arestrom I, Zuber B, et al. Nickel-induced IL-10 down-regulates Th1- but not Th2-type cytokine responses to the contact allergen nickel. *Clin Exp Immunol* 143:494–502, 2006.
26. Bellinghausen I, Konig B, Bottcher I, et al. Regulatory activity of human CD4+CD25+ T cells depends on allergen concentration, type of allergen and atopy status of the donor. *Immunology* 116:103–111, 2005.
27. Reefer AJ, Carneiro RM, Custis NJ, et al. A role for IL-10-mediated HLA-DR7-restricted T cell-dependent events in development of the modified Th2 response to cat allergen. *J Immunol* 172:2763–2772, 2004. □

Analysis of Helper T Cell Responses to Cry j 1-Derived Peptides in Patients with Nasal Allergy: Candidate for Peptide-Based Immunotherapy of Japanese Cedar Pollinosis

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ABSTRACT

Background: Allergen specific immunotherapy is highly effective, but adverse events may occur during treatment. Peptide-based immunotherapy has been proposed as one of new strategies for reduction of allergic adverse reactions. We examined the possibility of candidate peptides for the development of peptide-based immunotherapy for Japanese cedar pollinosis.

Methods: Twelve Cry j 1-specific T-cell lines were established from peripheral blood mononuclear cells (PBMC) of 12 patients with Japanese cedar pollinosis. Using these T-cell lines, 37 Cry j 1-derived overlapping peptides were assessed for their proliferative responses and cytokine production.

Results: Four peptides corresponding to the Cry j 1 sequence were able to induce proliferative responses to more than one T-cell line: p61-80 (3/12; 25.0%); p115-132 (2/12; 16.6%); p206-225 (4/12; 33.3%); and p337-353 (5/12; 41.7%). Furthermore, T-cell lines generated from 11 of 12 donors (91.7%) responded to at least one of these four peptides. On the other hand, the pattern of cytokine production from Cry j 1-specific T-cell lines varied. Moreover, cytokine production patterns by stimulation with Cry j 1 peptide did not reflect those by stimulation with Cry j 1 protein.

Conclusions: Our results suggest four Cry j 1-derived peptides (p61-80, p115-132, p206-225 and p337-353) may be considered to be the immunodominant T-cell epitopes of the Cry j 1 molecule, and can be useful for the design of peptide-based immunotherapy for the management of Japanese cedar pollinosis.

KEY WORDS

Cry j 1, immunotherapy, nasal allergy, peptide, Th response

INTRODUCTION

Allergic rhinitis is characterized by allergen-specific IgE production and activation of effector cells including eosinophils, mast cells, and basophils. These events are under regulation by a distinct subset of T lymphocytes, T helper 2 (Th2) cells that preferentially produce interleukin (IL)-4, IL-5, and IL-13. Thus, allergic diseases have been recognized as an

inadequate peripheral regulation of allergen-specific T-cells in individuals.

Allergen immunotherapy to allergic individuals induces a state of clinical and immunological tolerance; therefore, allergen immunotherapy is recognized as a highly effective treatment for allergic rhinitis. Evidence has accumulated indicating that immunotherapy inhibits allergen-driven Th2 responses by immune deviation in favor of Th1 responses and/or by

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Table 1 Clinical characteristics of the pollen allergic donors

Patients	Age/Sex	RAST score	Symptomatic period (m)	Other allergens ^a	Therapeutic history ^b
AR-1	27M	4	60	M, R	ME
AR-2	36M	2	24	—	None
AR-3	50F	2	12	—	ME
AR-4	38M	3	60	M	ME
AR-5	37M	3	36	M	ME
AR-6	37M	4	72	—	ME
AR-7	25M	2	60	M, R	ME
AR-8	34M	3	48	M	ME
AR-9	33M	4	72	—	ME
AR-10	32M	3	36	M	ME
AR-11	48F	3	72	R	ME
AR-12	16M	2	24	R	ME

^a M; house dust mites, R; ragweed.

^b ME; medications using oral antihistamines.

inducing T regulatory responses which produce the inhibitory cytokines such as IL-10 and transforming growth factor (TGF)- β .¹⁻³ Recently, intended as a novel strategy for allergen immunotherapy, the use of peptides which retain immunogenicity but are of insufficient length to cross-link IgE on the surface of mast cells, thereby reducing the risk of inducing anaphylaxis, has been introduced.^{4,5} Actually, Oldfield *et al.* have demonstrated that overlapping Fel d 1-derived T-cell peptides produced hyporesponsiveness to rechallenge with peptides as well as whole allergen, both clinically and in terms of *in vitro* T cell reactivity.^{6,7} Moreover, treatment using these peptides showed significant improvements in clinical outcome measurements such as the allergen-induced nasal and bronchial reactions, and asthma/rhinitis quality of life.⁸

Japanese cedar pollinosis caused by exposure to *Cryptomeria japonica* pollen is one of the most prevalent allergic diseases in Japan, and two major allergens, Cry j 1 and Cry j 2, have been isolated and specific IgE have been detected in patients suffering from pollinosis.^{9,10} To date, a number of HLA class II-restricted Cry j 1 and Cry j 2 epitopes defined by CD4+ T cell have been identified.¹¹⁻¹⁴ Among these epitopes, Hirahara *et al.* have reported the development of a hybrid peptide in which 3 dominant T-cell determinants in Cry j 1 and 4 in Cry j 2 have been considered to be potential therapeutic agents for Japanese cedar pollinosis.¹⁵ They chose p212-224, p235-247 and p312-330 from among T cell epitopes identified in the Cry j 1 sequence. Similarly, Sone *et al.* have also reported an artificial polypeptide which contains two (p108-120 and p211-225) and three T cell epitopes chosen from Cry j 1 and Cry j 2 sequences, respectively.¹² As expected, this polypeptide was able to stimulate more efficiently PBMC obtained from allergic patients as compared with individual peptides. To explore the possibility of other candidate

peptides for the development of peptide-based immunotherapy for Japanese cedar pollinosis, we investigated helper T cell responses to overlapping peptides derived from Cry j 1 sequence using Cry j 1-specific T cell lines obtained from patients with Japanese cedar pollinosis.

METHODS

SUBJECTS

Peripheral blood mononuclear cells were obtained from twelve patients whose diagnosis was based on clinical symptoms, positive skin prick tests, and radioallergosorbent test (RAST) scores against Japanese cedar pollen. The study was approved by the Institutional Review Board at the University of Yamanashi, University Hospital. Written informed consent was obtained from each individual. Details of the clinical data are shown in Table 1.

PURIFICATION OF Cry j 1 AND PEPTIDE SYNTHESIS

Cry j 1, a major allergen of *Cryptomeria japonica* (Japanese cedar) pollen, was purified as described.^{9,16} Briefly, dry pollen was defatted with ether, placed in 0.125 M ammonium bicarbonate, and stirred for 2 days at 4°C. Solid ammonium sulphate was added to the extract to 100% saturation. The precipitate was dissolved in 0.01 M Tris-HCL, pH 7.8, and dialyzed against the same buffer. A sample of this preparation was applied to a DEAE-cellulose column (Whatman, Madison, U.K.) and eluted with the same buffer. The eluate was dialyzed against 0.01 M acetate buffer (pH 5.0) and applied to a CM-cellulose column (Whatman). The column was eluted first with 0.01 M acetate buffer, and then with 0.1 M phosphate buffer, pH 7.0. The second elute was concentrated and purified by molecular sieving HPLC (Bio Sil TSK-250; BioRad, Hercules, CA). A fraction thus obtained contained 41 and 46 kDa proteins as determined by SDS-PAGE

Table 2 HLA class II genotypes of the pollen allergic donors

Patients	DR		DQ		DP
AR-1	DRB1 * 1403	DRB3 * 0301	DQA1 * 0501	DQB1 * 0301	DPB1 * 0202
	DRB1 * 1501	DRB5 * 0101	DQA1 * 0102	DQB1 * 0602	DPB1 * 0402
AR-2	DRB1 * 0405	DRB4 * 0101	DQA1 * 0301	DQB1 * 0401	DPB1 * 0501
	DRB1 * 0410	DRB4 * 0101	DQA1 * 0301	DQB1 * 0402	DPB1 * 0301
AR-3	DRB1 * 0101		DQA1 * 0101	DQB1 * 0501	DPB1 * 0501
	DRB1 * 1502	DRB5 * 0102	DQA1 * 0103	DQB1 * 0601	DPB1 * 0901
AR-4	DRB1 * 0405	DRB4 * 0101	DQA1 * 0301	DQB1 * 0401	DPB1 * 0501
	DRB1 * 0901	DRB4 * 0101	DQA1 * 0301	DQB1 * 0303	
AR-5	DRB1 * 0901	DRB4 * 0101	DQA1 * 0301	DQB1 * 0303	DPB1 * 0501
	DRB1 * 1502	DRB5 * 0102	DQA1 * 0103	DQB1 * 0601	DPB1 * 0901
AR-6	DRB1 * 0405	DRB4 * 0101	DQA1 * 0301	DQB1 * 0401	DPB1 * 0501
	DRB1 * 1401	DRB3 * 0202	DQA1 * 0101	DQB1 * 0502	
AR-7	DRB1 * 1403	DRB3 * 0101	DQA1 * 0501	DQB1 * 0301	DPB1 * 0201
	DRB1 * 1302	DRB3 * 0301	DQA1 * 0102	DQB1 * 0604	DPB1 * 0901
AR-8	DRB1 * 1405	DRB3 * 0202	DQA1 * 0101	DQB1 * 0503	DPB1 * 0202
	DRB1 * 0803		DQA1 * 0103	DQB1 * 0601	DPB1 * 0201
AR-9	DRB1 * 0405	DRB4 * 0101	DQA1 * 0301	DQB1 * 0401	DPB1 * 0201
	DRB1 * 1302	DRB3 * 0301	DQA1 * 0102	DQB1 * 0604	DPB1 * 0501
AR-10	ND		ND		ND
AR-11	DRB1 * 1502	DRB5 * 0102	DQA1 * 0103	DQB1 * 0601	DPB1 * 0402
	DRB1 * 1302	DRB3 * 0301	DQA1 * 0102	DQB1 * 0604	DPB1 * 0501
AR-12	DRB1 * 1502	DRB5 * 0102	DQA1 * 0103	DQB1 * 0601	DPB1 * 0201
	DRB1 * 1402	DRB3 * 0202	DQA1 * 0501	DQB1 * 0301	DPB1 * 0901

ND; not determined.

analysis (data not shown).

Peptides were synthesized from the amino acid sequence of Cry j 1 reported by Griffith *et al.*¹⁷ and Sone *et al.*,¹⁸ by utilizing a solid-phase simultaneous multiple peptide synthesizer PSSM-8 (Shimadzu, Kyoto, Japan) based on Fmoc strategy with PyBOP-HOBt-NMM (1 : 1 : 1.5). Peptides overlapping by 6 to 15 amino acids and covering the entire Cry j 1 molecule were produced.¹³ All peptides were purified by C18 reverse-phase high-performance liquid chromatography (Millipore Corp., Bedford, MA).

HLA CLASS II TYPING

Genotypes of HLA class II (DR, DQ, DP) alleles of the pollen allergic donors were determined by hybridization of HLA-DR, DQ, and DP genes amplified by polymerase chain reaction with sequence-specific oligonucleotide probes, as described previously.¹³

ESTABLISHMENT OF Cry j 1-SPECIFIC T-CELL LINES

T-cell lines were generated by stimulating 1×10^5 PBMC with Cry j 1 (2.5 µg/ml) in RPMI-1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% pooled, heat-inactivated normal human serum in each of sixty wells of 96-well flat-bottomed culture plates (Nunc, Roskilde, Den-

mark). After 7 days, irradiated (30 Gy) autologous PBMC at 1×10^5 /well, human rIL-2 (10 U/ml), human rIL-4 (10 U/ml), and Cry j 1 (2.5 µg/ml) were added to culture wells with T-cell blasts and maintained for an additional 7 days. Antigen-induced proliferation of the T-cell blasts was assayed, and the culture wells exhibiting Cry j 1-specific proliferation were transferred and propagated in a 24-well plate.

PROLIFERATION ASSAYS

T cells were co-cultured with irradiated (30 Gy) autologous PBMC (1×10^5 /well) in the presence of Cry j 1 (1.25 µg/ml) in 96-well flat-bottomed plates for 72 hours and pulsed with 1 µCi/well of [³H] TdR for the last 24 hours, and the incorporated radioactivity was measured by liquid scintillation counting. Stimulation indices (S.I.) were calculated by comparing the antigen-containing wells with those containing medium alone. In the case where synthesized peptides were used as antigen, PBMC (1.5×10^5 /well) were cultured with various overlapping peptides (2.5 µM) in culture medium for 2 hours at 37°C, after which excess peptides and nonadherent cells were removed by gently washing the plates three times with RPMI-1640 media containing 3% human serum. The remaining adherent cells were irradiated at 30 Gy and used as antigen presenting cells (APC).

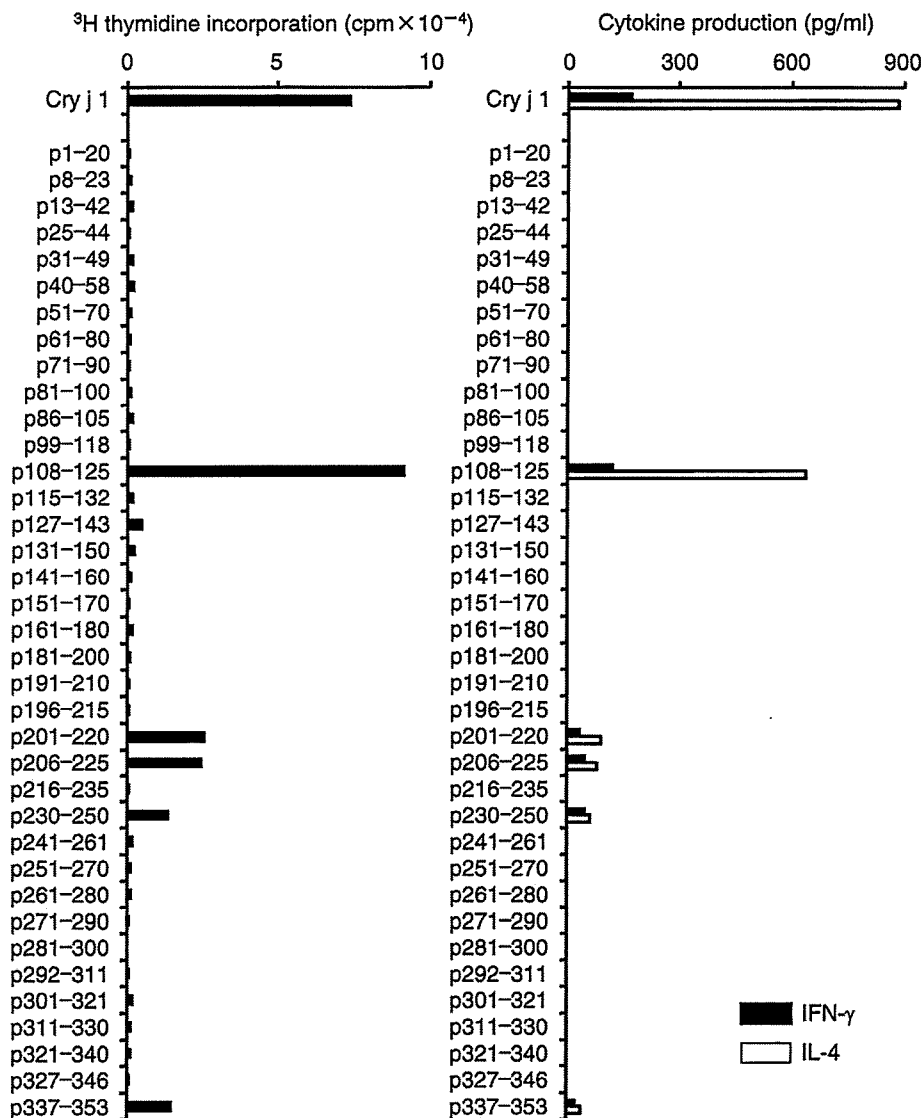


Fig. 1 Proliferative response and cytokine production by a Cry j 1-specific T cell line established from patient AR-1. Cry j 1-specific T-cell line established from patient AR-1 responded to 5 different peptides; p108-125, p201-220, p206-225, p230-250, and p337-353.

QUANTIFICATION OF IL-4 AND IFN-γ IN SUPERNATANT OF THE T CELL LINES

Culture supernatants of the same T cell lines evaluated for proliferative responses, after stimulation by APC plus antigen, were collected immediately before the addition of [³H] TdR and stored in aliquots at -80°C until the lymphokine concentrations were determined. IL-4 ELISA and IFN-γ ELISA (MEDGENIX, Fleurus, Belgium) were used to quantify IL-4 and IFN-γ in the supernatants according to the manufacturer's instructions.

RESULTS

ESTABLISHMENT OF Cry j 1-SPECIFIC T-CELL LINES

We generated Cry j 1-specific T-cell lines from PBMC obtained from all 12 pollen allergic donors (Table 1, 2). After 3 x in vitro stimulation, the outgrowing T cells were tested for their proliferative responses in the presence of Cry j 1 protein and irradiated autologous PBMC. Moreover, these Cry j 1-specific T cell lines were tested for their production of IFN-γ and IL-4. As expected, these T-cell lines produced various amounts of IFN-γ and/or IL-4 in response to Cry j 1 presented by autologous PBMC. Figure 1 shows the

Table 3 Proliferative responses to Cry j 1-derived peptides of Cry j 1-specific T-cell lines obtained from the pollen allergic donors^a.

Peptide	Patients												Total Responses
	AR-1	AR-2	AR-3	AR-4	AR-5	AR-6	AR-7	AR-8	AR-9	AR-10	AR-11	AR-12	
p13-32		++											1/12
p61-80			+							+		+++	3/12
p71-90								+					1/12
p81-100			+										1/12
p86-105			+										1/12
p108-125	+++												1/12
p115-132		+			+++								2/12
p127-143				++									1/12
p201-220	+++												1/12
p206-223	+++			+			+				+		4/12
p230-250	+++												1/12
p261-280			+										1/12
p271-290								+++					1/12
p301-321				++									1/12
p337-353	+++			++		+++			++		+		5/12

^a+: 5 ≤ S.I. < 50, ++; 50 ≤ S.I. < 100, +++; 100 ≤ S.I.

representative data obtained from patient AR-1.

PROLIFERATIVE RESPONSES TO Cry j 1-DERIVED OVERLAPPING PEPTIDES

Next, proliferative responses of each established T-cell lines against autologous PBMC pulsed with Cry j 1-derived overlapping peptides were investigated. As shown in Figure 1, Cry j 1-specific T-cell line established from patient AR-1 responded 5 different peptides; p108-125, p201-220, p206-225, p230-250, and p337-353. Similarly, all established Cry j 1-specific T-cell lines were tested for proliferative responses to Cry j 1-derived overlapping peptides. Table 3 summarizes the proliferative responses to Cry j 1-derived overlapping peptides. Among a panel of 37 overlapping peptides corresponding to the amino acid sequence of Cry j 1, 15 peptides were able to stimulate at least one T-cell line tested. These peptides represent a widely dispersed set of epitopes from the N- to the C-terminus of the Cry j 1 molecule. It is highly likely that these antigenic peptides include the T-cell epitopes of Cry j 1. Notably, 4 peptides were able to induce proliferative responses to more than two T-cell lines: p61-80 (3/12; 25.0%); p115-132 (2/12; 16.6%); p206-225 (4/12; 33.3%); and p337-353 (5/12; 41.7%). Furthermore, T-cell lines generated from eleven of twelve donors (all except AR-8) responded to at least one of these four peptides. Accordingly, these results suggest that these 4 peptides may be immunodominant T-cell epitopes of Cry j 1.

CYTOKINE PRODUCTION BY STIMULATION WITH Cry j 1 AND Cry j 1-DERIVED OVERLAPPING PEPTIDES

Nine of the 12 established T-cell lines were assessed for cytokine production by stimulation with Cry j 1 protein. However, the pattern of cytokine production from Cry j 1-specific T-cell lines varied. Two T-cell lines obtained from AR-1 and AR-2, produced IL-4 predominantly (Th2 pattern), whereas 4 (AR-6, -7, -8, and -9) produced IFN-γ predominantly (Th1 pattern), and 3 (AR-3, -4, and -5) produced IFN-γ and IL-4 equally (Th0 pattern), as shown in Table 4. Patients' RAST scores, the length of their clinical experience, and their therapeutic history did not appear to bear any relationship to their pattern of cytokine production (data not shown).

Next, the cytokine production by stimulation with 37 overlapping peptides was also assessed. Similar to proliferation responses, each T-cell line except AR-5, produced cytokines by stimulation with identical peptides that showed proliferative responses (data not shown). However, cytokine production patterns by stimulation with Cry j 1 peptide did not reflect those by stimulation with Cry j 1 protein (Table 4). Although most T-cell lines reactive with Cry j 1-derived peptides produced some IL-4 irrespective of the amount of IFN-γ production. Interestingly, however, the T-cell lines obtained from patients AR-3 and AR-8 secreted IFN-γ but failed to produce IL-4 in recognition of the peptides, p86-105 and p271-290, respectively. For further characterization of these two peptide specific T-cell lines, these T-cell lines were incubated with peptide-pulsed APC during various peri-