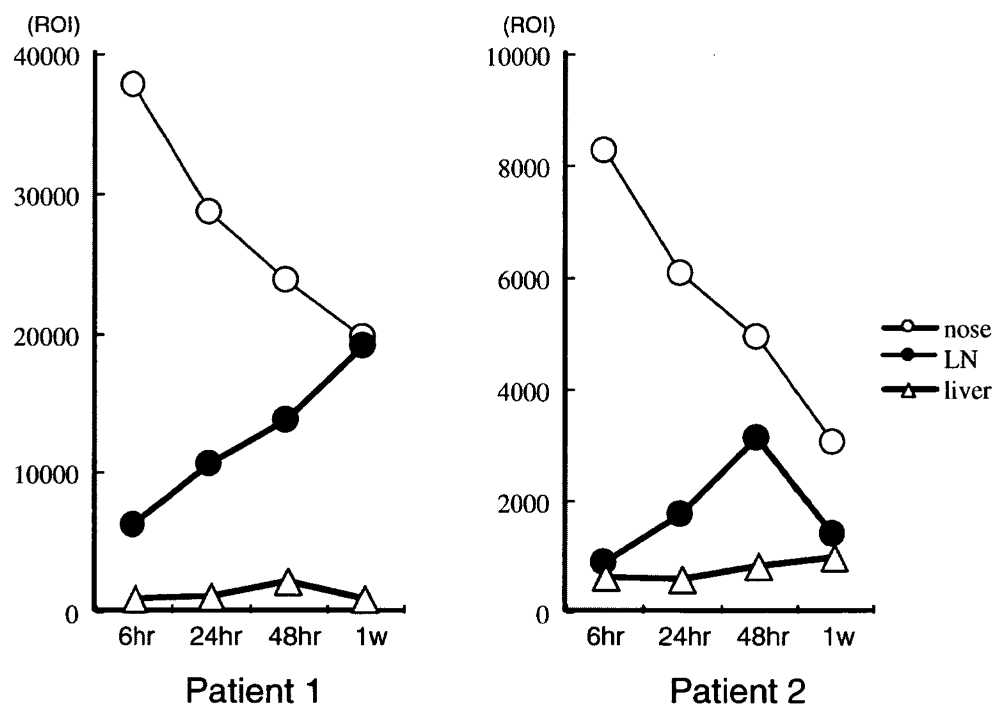

Fig. 3 The isotope counts in the ROI analysis of the neck, nose, and liver after the labeled DCs injection into the nasal mucosa. Although the isotope counts in the nose rapidly decreased, the peak count in the neck was observed 48 h or after. The count in the liver was lower.

In-111 Labeling of DC

Indium-111-Oxine(^{111}in) is used widely as a diagnostic radiopharmaceutical for radiolabeling autologous leukocytes (white blood cells). DC labeled with ^{111}In are routinely used to detect immunological events associated with antigen presentation. The labeling of DC with ^{111}In was done according to the manufacturer's protocol (Amersham Co. Ltd., UK). The SART-1 peptide-loaded DCs were resuspended in 10 ml phosphate-buffered saline (PBS) containing 37 Mbq ^{111}In ($T_{1/2}$=2.8005 days). After incubation for 15 min, the DCs were washed with normal saline containing 5% autologous serum. The labeling efficacy was determined by measuring the radioactivity of the cellular fraction in comparison to that of the supernatant, and it was usually found to be more than 80%.

Administration of Labeled DC

Three patients received an injection of 10×10^6 ^{111}In-labeled DCs in 200 μl of PBS submucosally into the inferior turbinate. Two patients received the same number of the DC via injection into the palatine tonsil. The remaining two patients received the DC intravenously (Table I). Total body image and single photon emission computed tomography (SPECT) images were performed 6, 24, 48 h and 7 days after the administration of the labeled DC to determine the kinetics of the DC migration. An image analysis was performed using a region-of-interest (ROI) analysis of the regional lymph nodes, using the liver as a control, to obtain decay-corrected counts and geometric means for the anterior and posterior views.

Detection of Tumor Antigen Specific Cytotoxic T Cells in the Neck Lymph Nodes

Four patients received the unlabeled DCs by nasal mucosal injection, and 7 to 10 days later, specimens of neck nodes in the neck were obtained after surgical dissection. The tumor antigen-specific activating IFN-γ secreting cells were identified by an enzyme-linked immunosorbent spot (ELISPOT) assay using the surgically obtained lymph nodes. After cutting the nodes into small pieces, lymphocytes were obtained with a cell strainer using the Ficoll–Hypaque technique. The lymphocytes were then washed and transferred 1×10^5 cells to an ELISPOT assay kit (BD Pharmingen) with 96-well filtration plates coated with anti-IFN-γ capture antibody (Mabtech Co. Ltd., Stockholm, Sweden) for 16 h. The stimulation conditions were 100 ng/ml of SART peptides in AIM-V. After extensive washing with PBS, biotinylated anti-human IFN-γ antibody was added. Two hours later, the spots were detected by an avidin biotin-peroxidase complex and aminoethyl carbazole solution. The mean values of the spots in three wells were utilized for subsequent analysis. The statistical analysis was done using a Wilcoxon-signed rank test for paired and unpaired data.

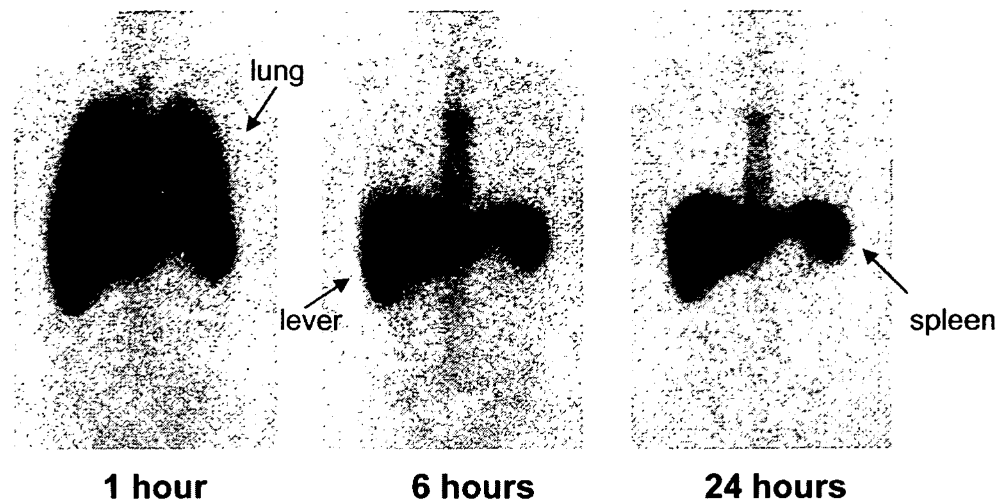

Fig. 4 An anterior image of chest recorded 1, 6, and 24 h after the intravenous injection of labeled DCs. The intense DC spots were observed in the lung and liver 1 h after injection, but the spots in the lung were very weak 6 h thereafter and then were not detected 24 h later.

Results

Using flow cytometry, the DCs were characterized by the expression of HLA-DR and by the lack of expression of CD3, CD14, CD19, and CD56 markers (data not shown). The DC represented an activated phenotype, as reflected by their increased expression of major histocompatibility complex class II (MHC II) molecules and costimulatory CD80 and CD86 molecules and by the presence of activation marker CD83 (Fig. 1).

After the administration of antigen-pulsed DCs, two patients developed transient mild fever, but no other symptoms or major physical abnormalities were detected. No major toxicity (above Common Terminology Criteria for Adverse Event-grade 2) was observed in any of the patients studied.

In the three patients who received labeled DCs via an injection into the nasal mucosa of the inferior turbinate, DC spots were observed at the primary injection sites, ipsilateral to the upper neck lymph nodes. An anterior image (Fig. 2a) and a SPECT image of a sagittal section (Fig. 2c) were recorded for 48 h after the injection of DCs into the left nasal inferior turbinate. The DCs spots were detected with high intensity in the neck region. The administered DCs migrated to the neck but did not spread within the nasal mucosa. An anterior image (Fig. 2b) of the head and neck area 48 h after injection and a SPECT sagittal section image (Fig. 2d) of the two patients who received labeled DCs through injection into the palatine tonsils are shown. The DCs remained in the injected tonsils and did not migrate to the neck lymph nodes or to the nasal mucosa.

The radioactivity counts in the neck, nose, and liver for the two patients who received labeled DC via nasal mucosal injection are shown in Fig. 3. Peak radioactivity was observed at 48 h. Although the isotope counts in the nose decreased rapidly, some activity was observed in the neck lymph nodes even 6 h after administration of labeled DC. The counts in the liver were lower and the peak obscure.

When the labeled DC were administered intravenously, the DC spots were observed in the lung, liver, and spleen 1 h after injection but were detected only rarely 6 h later. The DC spots were detected regularly in the liver and spleen 24 h later (Fig. 4). No DC spots were observed in the neck or in the upper respiratory mucosa.

Figure 5 shows the IFN-g secreting cells in the upper neck lymph nodes 5 days after the administration of the SART-peptides-pulsed DCs into the nasal submucosa.

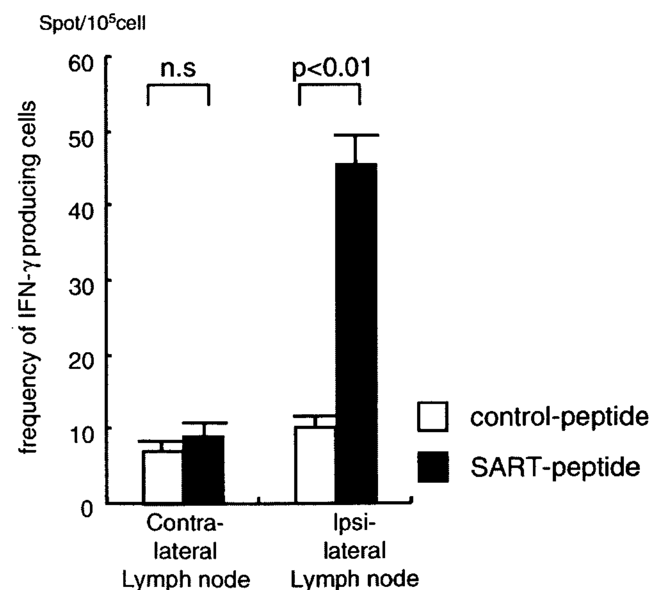

Fig. 5 IFN-g-producing cells in the upper neck lymph nodes after the administration of the SART peptides pulsed DCs into the nasal submucosa. SART-specific IFN-g-producing cells were detected in the ipsilateral lymph nodes of the administered DCs but not in the contralateral lymph nodes 5 days after administration. The data was expressed as mean±SD of the detected spots in each two lymph nodes of ipsilateral and contralateral sides from four patients.

SART peptides specific IFN-γ secreting cells induced by the DCs were detected only in the ipsilateral neck lymph nodes after the DCs administration but not in the contra lateral lymph nodes after 5 days.

Discussion

In this study, we examined the migration of isotope-labeled tumor antigen-specific peptide-pulsed DC after administration in the upper respiratory mucosa. The DCs administered to the palatine tonsils remained at the injection site in the tonsils and did not migrate to the neck lymph nodes. These observations suggest that human tonsils serve as an inductive site of mucosal immunity. An earlier study on the intratonsillar immunization with cholera and tetanus toxoids has shown the induction of the antibody secreting cells in the tonsils and specific IgA and IgG antibody response in the nasal wash and in the serum [20]. These observations support the possibility that human tonsils support the development of immunologic memory and serve as an inductive site for mucosal immune responses.

In the present studies, it was observed that the DCs administered into the nasal submucosa quickly migrated to the regional neck lymph nodes of ipsilateral sides of the administration, and this migration increased over time, but spreading within the nasal mucosa was not observed. These DCs also tended to remain in the neck and, after migration, induced the tumor-antigen-specific IFN-γ secreting cells in the neck lymph nodes in the ipsilateral sides of the DCs administration but not on the contra lateral sides where labeled DCs spots were not observed.

The migration of DCs, upon the uptake of antigens, from peripheral tissues to the regional draining lymph nodes is considered to be an important immunological function of such cells. However, the migration of DCs in the upper respiratory tract has not been studied extensively in man. The observations reported in this study suggest that the major area of antigen presentation and of induction of memory cells or effector cells may be in the regional neck lymph nodes in humans and not in the nasal mucosa. Most head and neck cancers arise from the upper respiratory or digestive mucosa, and neck lymph nodes tend to normally be defined as regional. The submucosally administered DCs are thus expected to migrate to the regional lymph nodes, thereby effectively inducing mucosal anti-tumor responses. The actual induction of tumor antigen-specific IFN-γ secreting cells in the neck lymph nodes after tumor antigen-pulsed DCs administration into the nasal submucosa observed in this study may have therapeutic implications in the specific immunologic treatment of some head and neck cancers. However, DC migration is known to be affected by various proinflammatory and anti-inflammatory mediators [21, 22]. In addition, cancer cells could produce factors, which would affect DC migration [23]. The influence of these mediators and of pulsed tumor antigen on DC migration need to be studied in further detail.

Conclusions

In summary, the tumor specific-antigen-pulsed DCs administered into the nasal submucosa of the patients with head and neck cancer quickly migrated to the regional neck lymph nodes and induced the tumor antigen-specific IFN-γ secreting cells. Further studies should be carried out to examine the antigen-processing phenomenon in the nasal mucosa and the distribution of the CTLs or effector cells in the nasal mucosa and tonsils and the cancer lesions after the administration of antigen-pulsed DCs.

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Short communication

Detection of natural killer T cells in the sinus mucosa from asthmatics with chronic sinusitis

Background: Chronic sinusitis (CS) with asthma generally exhibits a high degree of sinus tissue eosinophilia and recurrence often occurs even after surgical therapy. However, the cause has not yet been fully clarified.
Aims of the study: To elucidate the pathogenesis of this refractory disease, we examined the infiltration of natural killer T (NKT) and type 1 helper T (Th1)/type 2 helper T (Th2) cells, and the cytokine expression in the sinus mucosa.
Methods: Sinus mucosal specimens were obtained surgically from 16 CS patients with nasal polyps. The NKT cells, Th1/Th2 cells and the expression of IL-4, IL-5, IL-13 and IFN-γ were examined by a polymerase chain reaction or flow cytometry. Nasal mucosal specimens from six other patients with allergic rhinitis (AR) were examined in a similar manner.
Results: The NKT cells were detected to varying degrees in the sinus mucosa from asthmatic CS patients, but neither in the nonasthmatics nor in the nasal mucosa from the patients with AR. The Th2 cells and Th2 cytokines were expressed at significantly higher levels in the sinus mucosa from the CS patients with asthma in comparison to those without asthma. However, the Th1 cell infiltration and IFN-γ expression were not different between these groups.
Conclusion: Natural killer T cells may, therefore, play important roles in the enhanced Th2 cytokine expression and increased infiltration of Th2 cells and eosinophils observed in the sinus mucosa from asthmatic CS patients through MHC-independent mechanisms.

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Key words: allergic rhinitis; asthma; chronic sinusitis; eosinophil; natural killer T cells.

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Chronic sinusitis (CS) is a common disease that affects about 20% of the population, and is usually treated by conservative medical management (1). Many cases of CS with polyps in Japan predominantly exhibit an infiltration of neutrophils in the sinus and are generally responsive to macrolide therapy (2, 3). Although some cases of CS with sinus tissue neutrophilia (CSN) require surgical intervention, their prognosis is generally satisfactory (1, 4).

However, there is a severe subform of CS, which exhibits marked sinus infiltration with eosinophils and, to a lesser degree, neutrophils and other infiltrating cells. These cases of CS with sinus tissue eosinophilia (CSE) also exhibit the characteristics of multiple nasal polyps, relapse even after surgery, and a high degree of complication with asthma (5, 6). Although the involvement of fungal infection (7) and some bacterial toxins as allergens or superantigens has been reported (8), the exact mechanisms of development of CSE have not yet been ascertained. The involvement of a type 1 allergic reaction in CSE remains uncertain because it occurs infrequently with allergic rhinitis (AR) and CSE is more frequently linked to nonatopic rather than atopic asthma (9–11).

Human natural killer T (NKT) cells express the invariant Vα24JαQ paired with the Vβ11 antigen receptor and play important roles in innate immunity (12). Natural killer T cells are activated by a specific glycolipid antigen, α-galactosylceramide (α-GalCer), in a CD1d-dependent manner (13). CD1d is an HLA class I-like antigen priming molecule that does not exhibit any allelic polymorphism. Therefore, the NKT activity is not restricted to MHC antigens. Recently, the involvement of NKT cells in the development of airway hypersensitivity in mice (14) and the detection of NKT cells in bronchoalveolar-lavage fluid samples from patients with moderate to severe asthma (15) were reported.

In the present study, we examined NKT cells, as well as Th1/Th2 cells and the expression of various cytokines in

Abbreviations: α-GalCer, α-galactosylceramide; AR, allergic rhinitis; CS, chronic sinusitis; CSE, chronic sinusitis with sinus tissue eosinophilia; CSN, chronic sinusitis with sinus tissue neutrophilia; NKT cell, natural killer T cell.

the sinus mucosa from asthmatic and nonasthmatic CS patients.

Methods

Patients and samples

All patients included in this study complained of CS with nasal polyps, were operated at Chiba University Hospital from January to October 2006. Chronic sinusitis was clinically determined as the presence of a combination of major symptoms (such as nasal discharge, nasal obstruction, hyposimia, facial congestion, facial fullness) and computed tomography (CT) findings. The symptoms were scored in all patients. The symptom scoring range in this study had four options as reported previously (16, 17). The exclusion criteria included cystic fibrosis, immotile cilia syndrome, autoimmune diseases and being positive response to HIV, hepatitis C virus and for hepatitis B surface antigens. The diagnosis of asthma was based on the American Thoracic Society criteria (18) and patient with aspirin-intolerant asthma was proven to have histories of severe bronchoconstriction after the ingestion of at least two different nonsteroidal anti-inflammatory drugs.

All patients received various medications including anti-histamines, anti-leukotrienes, and local or systemic steroids but failed to show any significant improvements. All medications were withdrawn for 1 week prior to the operation, however, the one patient (#15 in Table 1) could not stop using oral steroids because of asthma. A preoperative examination included allergen specific IgE (mite, cedar, house dust, Candida, Alternaria by CAP radio-allergo sorbent test) and blood eosinophil counts by using a standard automated cell counter. Surgical biopsy samples from the ethmoidal sinus mucosa, including polyps, were obtained and examined for inflammatory cells by hematoxylin and eosin staining. The sinus mucosa specimens from each patient were immediately cut into small pieces and incubated with PBS containing 5% fetal calf serum (FCS), 1 mg/ml collagenase A (Roche, Mannheim, Germany) and 0.2 mg/ml DNase I (Sigma-Aldrich, St Louis, Mo, USA) at 37°C for 35 min. After incubation with 20 mM EDTA for 5 min at room temperature, the specimens were passed through a fine nylon mesh. Mononuclear cells were subsequently obtained by the Ficoll–Hypaque technique and were used for the polymerase chain reaction (PCR) assay. For the flow cytometric analysis, collagenase and DNase I were not used during the preparation of the mononuclear cells to avoid interference with the cell surface markers.

Another six patients with serious perennial AR due to *Dermatophagoides pteronyssinus* were also enrolled in this study. The diagnosis of AR was based on the criteria of Okuda et al. (19), namely, typical perennial allergic nasal symptoms and a positive CAP radio-allergo sorbent test

Table 1. Profile of chronic sinusitis (CS) and allergic rhinitis (AR) patients

	Patient	Age	Sex	Blood eosinophils (%)	AR	CS with polyps	Tissue eosinophils*	Tissue neutrophils*	E/N ratio†	CS Score‡
CS without asthma	1	54	M	n.t.	–	+	5	90	0.06	2.6
	2	56	M	1.1	–	+	4	120	0.03	2.4
	3	55	M	2.6	–	+	6	180	0.03	2.6
	4	77	M	1.0	–	+	2	110	0.02	2.6
	5	38	M	3.7	–	+	12	400	0.03	2.8
	6	65	M	2.5	–	+	3	140	0.02	2.6
	7	71	F	1.8	–	+	2	70	0.03	2.8
	8	64	M	n.t.	–	+	3	120	0.03	2.4
	9	32	M	3.4	+	+	6	150	0.04	2.6
	10	57	M	6.6	+	+	135	50	2.7	2.8
CS with asthma	11	43	F	1.8	–	+	42	120	0.35	2.8
	12	61	M	11.2	–	+	>500	110	>4.5	3
	13	25	M	4.7	–	+	125	50	2.5	2.6
	14	57	F	16.9	–	+	190	55	3.5	2.8
	15	45	M	2.6	–	+	330	80	4.1	2.6
	16	56	F	8.0	–	+	>500	100	>5.0	2.8
AR	17	53	M	4.9	+	–				
	18	66	M	7.1	+	–				
	19	25	F	14.7	+	–				
	20	37	M	7.1	+	–				
	21	54	F	n.t.	+	–				
	22	22	M	4.9	+	–				

n.t., not tested.
*Number of eosinophils and neutrophils/×400 magnification.
†Number of tissue eosinophils/number of tissue neutrophils.
‡Severity scoring of CS major symptoms (from ref. 17).

result against *D. pteronyssinus*. The inferior nasal turbinate mucosa was obtained surgically and was examined in the same manner.

The study received prior approval from the Ethics Committee of the Chiba University. Informed consent was obtained from each patient.

PCR analysis

Total RNA was isolated from the mononuclear cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized using oligo primers and Superscript II RT (Invitrogen). The specific primers for the detection of IL-4, IL-5, IL-13, IFN-γ CD3, GAPDH were purchased from Applied Biosystems (Foster City, CA, USA). For the detection of Vα24Jα18, the forward primer 5′-CCTCCCAGCTCAGCGATTC-3′, the reverse primer 5′-TATAGCCTCCCCAGGGTTGA-3′ and the probe FAM-5′-CCTCCTACATCTGTGTGGTGAGC-GACA-3′-TAMtph were used (20). The samples were subjected to a real-time PCR analysis on an ABI PRISM7000 Sequence Detection system.

Flow cytometric analysis

The mononuclear cells were incubated on ice with the appropriate staining reagents: fluorescein isothiocyanate (FITC)-conjugated anti-CD4 monoclonal antibody (mAb), R-phycoerythrin (R-PE)-conjugated anti-CCR4 mAb, allophycocyanin (APC)-conjugated anti-CXCR3 mAb, or FITC-, RPE- or APC-conjugated isotype match control mAb (BD Bioscience, San Jose, CA, USA) (21).

The cells were subjected to a flow cytometric analysis using a flow cytometer (FACS Calibur: BD, Franklin Lakes, NJ, USA) and the results were analyzed using the FlowJo software program (Tree Star, Inc, Ashland, OR, USA).

Statistical analysis

A statistical analysis was performed using the Wilcoxon rank sum test or the Wilcoxon signed rank test for paired and unpaired data. *P*-values < 0.05 were considered to be statistically significant.

Results

A total of 16 patients were analyzed. All patients had a severe loss of smell and nasal obstruction. Allergic fungal sinusitis with eosinophil granule-rich mucin and diagnostic clinical characteristics (17) was not detected in these patients. Six patients had asthma and two of them were aspirin-intolerant asthma (#12, 16 in Table 1). All these asthma patients were negative for specific IgE to inhalant allergens.

Based on the number of sinus mucosal eosinophils, the CS patients were divided into CSE [number of eosinophils/number of neutrophils (E/N ratio) > 1.0] and CSN (E/N ratio < 1.0). Of the 16 patients with CS, six were considered to be CSE and ten were CSN. Five of the six CSE patients had asthma and one had AR but not asthma. Of the 10 patients with CSN, one had asthma and one had AR.

IL-5 mRNA expression was observed in the mononuclear cells isolated from the sinus mucosa from all patients with asthma or with CSE and the expression was significantly higher than in patients without either asthma or with CSN. These findings were similar to the expression of IL-4 and IL-13 (Fig. 1). Although IFN-γ was detected in the sinus mucosa from each of the patients, there was no significant difference between patients with and without asthma (data not shown).

In the flow cytometric analysis of the sinus mucosa, the number of CXCR3-positive T cells was not different

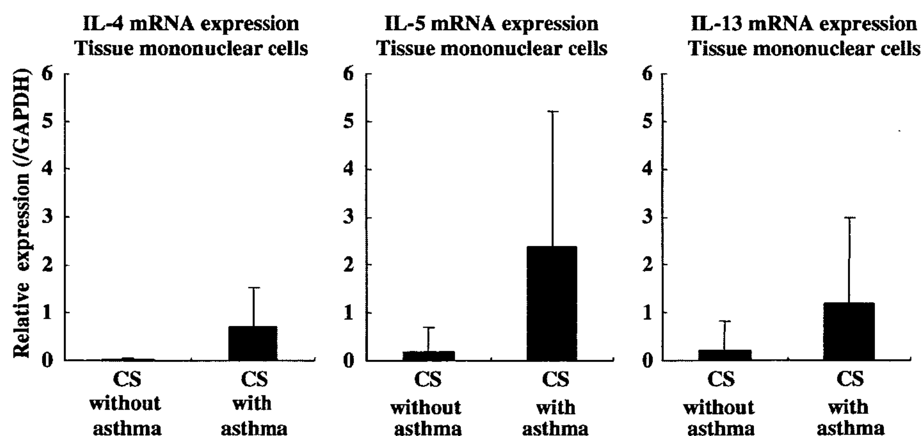

Figure 1. IL-4, IL-5 and IL-13 mRNA expression in mononuclear cells isolated from sinus mucosa in chronic sinusitis patients. Significantly increased expression was observed in patients with asthma by real time PCR. This indicated that the T cells produced significant levels of IL-4, IL-5 and IL-13.

Figure 2. The ratio of CXCR3-positive and CCR4-positive T cells to CD4-positive T cells in mucosal specimens from chronic sinusitis (CS) patients with or without asthma, or in nasal mucosa from patients with allergic rhinitis (AR). Although the ratio of CXCR3-positive T cells were not different among these groups of patients, CCR4-positive T cells were increased significantly in asthmatic CS and AR patients compared with the nonasthmatic CS patients.

between the patients with and without asthma, nor between CSE and CSN. However, a significantly greater number of CCR4-positive T cells detected in patients with asthma or with CSE in comparison to those without either asthma or with CSN (Fig. 2).

NKT cells were detected at various levels in the peripheral blood of all CS patients, however, in sinus mucosa NKT cells were only detected in the patients with asthma. Five out of six patients with CSE and only one patient with CSN exhibited NKT cells in the sinus mucosa (Fig. 3). The patient with CSE who did not exhibit NKT cells in the sinus mucosa had perennial AR due to mites, but not asthma. The patient with CSN exhibited NKT cells in the sinus mucosa, combined with asthma, and an expression of Th2 cytokines and infiltration of CCR4-positive T cells was observed in the sinus mucosa. The expression levels did not correlate with the severity of CS.

In the inferior nasal turbinate mucosa from the six patients with AR, the CCR4-positive T cells were as high as those in the CSE patients, and the CXCR3-positive T cells were similar to those of CSN and CSE. However, NKT cells were not detected in the nasal mucosa from any of the patients with AR (Fig. 3).

Figure 3. The relative expression of natural killer T (NKT) cells to CD3 T cells. NKT cells were detected in the sinus mucosa from five of six chronic sinusitis with sinus tissue eosinophilia (CSE) patients and one of 10 chronic sinusitis with sinus tissue neutrophilia (CSN) patients. The one CSE patient (indicated by the arrow) who was negative for NKT cells did not have asthma, but exhibited allergic rhinitis. The single CSN patient (indicated by the arrow) who was positive for NKT cells had asthma.

Discussion

In the present study, a significantly more number of Th2 cells, and consequently, expression of Th2 cytokines, was observed in the sinus mucosa from patients with asthma or with CSE. The low level of expression of Th2 cytokines and infiltration of Th2 cells in the sinus mucosa among the patients without asthma or with CSN, along with the fact that there was no difference in the Th1 cell infiltration among these sinus mucosa, altogether indicate that the Th2-predominant state in the sinus mucosa is characteristic of CS with asthma and of CSE.

A similar Th2-predominant state was observed in the nasal mucosa from patients with AR, which is a typical type I allergic disease mediated through allergen-specific IgE antibodies, although the pathogenesis of CSE is considerably different. The complication rate of AR in CSE is not high, and highly complicated asthma with CSE is nonatopic rather than atopic (9–11).

In this study, we detected NKT cells at various levels in the PBMCs from all patients, with no significant difference between the CSE and CSN patients. In the sinus mucosa, NKT cells were recognized in all the patients with asthma, in five out of the six patients with CSE and

in one CSN patient. The patient with CSN who was positive for NKT cells in the sinus mucosa had asthma and exhibited some degree of infiltration of eosinophils and Th2 cells, despite the predominant infiltration of neutrophils. In contrast, the patient with CSE who was negative for NKT cells in the sinus mucosa had AR and a Th2-predominant state in the sinus mucosa, but this was not combined with asthma. Natural killer T cells were not detected in the sinus mucosa from the CS patients without asthma, or in the nasal mucosa from the AR patients. These results suggest that NKT cells are not directly related to the development of allergy, but they may play important roles in the development of sinus disease combined with asthma as 'one airway, one disease (22)', and substantially different mechanisms must exist between allergy and the accumulation of eosinophils in the sinus mucosa in CSE. In a recent study with mice, Meyer et al. (23) demonstrated eosinophil accumulation in the airway and the development of airway hyperreactivity following the respiratory administration of α-GalCer, a specific NKT cell-activating glycolipid antigen, and that eosinophil accumulation was observed in the respiratory tract even in MHC class II-deficient mice. In the present study, we clearly demonstrated the presence of NKT cells in the sinus mucosa from CS patients with asthma, but not from CSE patients without asthma, nor in the nasal mucosa from AR patients. Chronic sinusitis with sinus tissue eosinophilia may be composed of several diseases with different etiologies. Natural killer T cells were detected in the sinus mucosa from CS patients with asthma and are, therefore, suggested to participate in the local immune reactions of the sinus mucosa. The elucidation of the mechanisms of NKT cell activation in the sinus mucosa and its relationship to the process of tissue eosinophilia may, therefore, lead to the fundamental resolution of these refractory diseases, including asthma.

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ORIGINAL PAPER Asthma and Rhinitis

Seasonal changes in antigen-specific T-helper clone sizes in patients with Japanese cedar pollinosis: a 2-year study

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Summary

Background Allergic rhinitis (AR) is a typical type I allergic disease that occurs through the induction of allergen-specific effector T cells. Once established, new effector T cells derive mostly from memory T cells that are capable of surviving for extended periods, although the mechanisms by which these memory functions are maintained have not yet been clarified. In particular, the exact life-span of memory T cells is still not well understood.

Objective Pollinosis patients seemed to be suitable subjects to investigate because such patients are exposed to antigens strongly for only a limited period once a year. We compared the seasonal changes in memory T-helper type 2 (Th2) between pollinosis and perennial allergic subjects.

Methods The clone sizes of the Japanese cedar pollen-specific memory Th cells were measured by an ELISPOT assay using specific peptides from the patients with cedar pollinosis, and the seasonal changes were noted. This study was performed for 2 years. The cedar-specific IgE levels in the peripheral blood were also studied. Mite allergy patients were also enrolled in the study.

Results The Japanese cedar-specific IL-4-producing Th2 cells were detected in all patients examined, although the number of cells was low. These Th memory cells increased during the pollen season and decreased during the off-season. However, more than 60% of the cedar-specific memory Th2 cells survived up to 8 months after the pollen season. The cedar-specific IgE levels exhibited changes similar to the cedar-specific Th cells. On the other hand, there was no drifting of Th memory clone size with the mite allergics, and the IgE levels also did not change.

Conclusions While pollen-specific Th cells decreased after pollen exposure, their memory functions continued. Memory clone size maintenance therefore requires repetitive antigen irritation.

Keywords allergic rhinitis, clone size, IgE, memory T cell, Th2

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Introduction

In recent years, many countries have experienced an increase in the prevalence of allergic rhinitis (AR) [1, 2]. In Japan, Japanese cedar (*Cryptometria japonica*) and Japanese cypress (*Chamaecyparis obtusa*) pollens are considered to be the major unique allergens and their extent of dispersal is quite large, travelling more than 100 km and thus causing serious pollinosis [3, 4].

Pollinosis is thought to be an adaptive immune response that manifests as a type I allergic reaction, and it occurs as a consequence of fundamental allergenic mechanisms involving the induction of pollen-specific T-helper type 2 (Th2) effector cells from naïve Th0 cells [5]. Most effector T cells are short-lived, but few effector T cells become long-lived memory T cells. Once a memory T cell is established, it retards the induction of new effector T cells from naïve Th0 cells, according to the principle of the 'original antigenic sin'. Hence, most effector T cells are derived from memory T cells [6–10]. This concept describes a phenomenon in which the antibody response elicited in an individual after a secondary viral infection reacts more strongly to the viral variant that originally infected the individual. A similar phenomenon

is likely to be observed in Th cell responses as they play critical roles in promoting antibody responses.

Patients with type I allergy are thought to have allergen-specific memory Th cell clones. Immune-therapeutic intervention directed at diminishing the size of these clone memory Th2 cells and shifting the cytokine type of memory Th clones is thought to play a considerably important role in finding a complete cure.

However, the mechanism by which the antigen-specific Th memory clone is maintained in an allergic patient has not yet been clarified. There are several reports describing the peripheral blood to be re-stimulated by a specific antigen, while the Th2 cytokine response was measured both in season or/and out of season [11–13] as well as before and after immunotherapy [14–16]. In those studies, peripheral blood mononuclear cells (PBMCs) were stimulated by an antigen, and measured Th2 cytokine or its mRNA. However, those cytokine responses that were re-stimulated by whole antigen reflected various kinds of cells such as T cells, B cells, macrophages and antigen-presenting cells and did not precisely reflect the function or the exact number of antigen-specific Th cells. Because the Th cell response is restricted in major hitocompatibility complex class II, it is necessary to use the Th cell epitope of class II restrictive to measure the reaction only for Th cell clones respond to allergen. Our purpose is to estimate the duration of life-span of allergen-specific memory T cells. We therefore directly examined the number of specific Th2 cells to respond to class II restrictive T cell epitope, which matched with Japanese human leukocyte antigen (HLA) variation. These kinds of studies have not yet been carried out. Pollinosis seemed to be a suitable subject to investigate the life-span of Th memory clones because patients are exposed to the antigen for only a limited period. In the present study, we examined the specific memory clone size, which is a population size of memory T cells that recognizes the same specific HLA restrictive epitope and produces isologous cytokine. We tried to detect IL-4 productive cells using only seven T cell epitopes; as a result, the total summation of these seven kinds of clones is the clonal size.

The limited seasonal nature of antigen exposure is useful for elucidating the mechanisms used in the maintenance of Th memory clone sizes. We examined the Japanese cedar pollen-specific Th clone sizes and the associated seasonal changes in patients with cedar pollinosis, while also comparing the yearly change in the clone size due to pollinosis with that due to perennial mite allergies that were detected by 14 T cell epitopes.

Materials and methods

A total of 41 patients with Japanese cedar pollinosis were enrolled in this study. The ages of the 20 males and 21 females ranged from 20 to 51, with an average of 31.1 years. The diagnosis of Japanese cedar pollinosis was based on the occurrence of typical nasal symptoms during the cedar pollen season and the detection of Japanese cedar-specific IgE by CAP-RAST (score: 2 or more). All patients had symptoms for at least 3 years. None of the patients received immunotherapy or immunosuppressive drugs (including steroids) within 8 weeks before the start of the study. The study received prior approval from the Ethics Committee of the Chiba University (Chiba, Japan). A written, witnessed informed consent was obtained from all patients. The study design is shown in Table 1. From 2003 to 2004, 23 patients participated in this study, and from 2004 to 2005, another 18 patients were enrolled. Twenty-two patients with perennial AR due to mite were also enrolled in this study. The ages of the 10 males and 11 females with perennial AR were from 20 to 48, with an average of 28.8 years. The diagnosis of mite allergy was based on the occurrence of typical nasal symptoms and the detection of Der f IgE by CAP-RAST (score: 2 or more).

Ten healthy subjects were also enrolled as controls. They were all negative for symptom episodes and allergen IgE.

The blood samples were collected every 3 months after July, and the PBMCs were obtained by the Ficoll–Hypaque method from the patients. The samples were stored in liquid nitrogen until analysis.

Clinical symptoms

The nasal symptoms were evaluated on a scale from 0 to 4 in accordance with the practical guidelines for the treatment of AR [17], as follows: 0, no sensation; 1, mild; 2, moderate; 3, severe; and 4, extremely severe. Daily episodes of sneezing and nose blowing were rated 0 to 4, as follows: 0, none; 1, 1–5 episodes; 2, 6–10 episodes; 3, 11–20 episodes; and 4, >20 episodes. The medication was also recorded according to drug characteristics and duration of usage, according to the guidelines, as follows: antihistamine, mast cell stabilizers and vasoconstrictor were 1, and topical ocular or nasal steroids were 2.

Reagents

Antibodies. The monoclonal antibodies (MoAb) used for the ELISPOT assay were acquired from MABTECH (Stockholm, Sweden). For coating, MoAb 82-4 and MoAb 1-DIK were used to coat human IL-4 and IFN-γ, respectively. For detection, MoAb 12-1 and 7B6-1 were used to detect human IL-4 and IFN-γ, respectively. For the FACS analysis, anti-human Cy5-conjugated CD4, FITC-conjugated IFN-γ and PE-conjugated IL-4 were purchased from Dako (Tokyo, Japan).

Peptides. A recombinant hybrid peptide was used for the ELISPOT assay. This peptide comprised of the seven CD4 T

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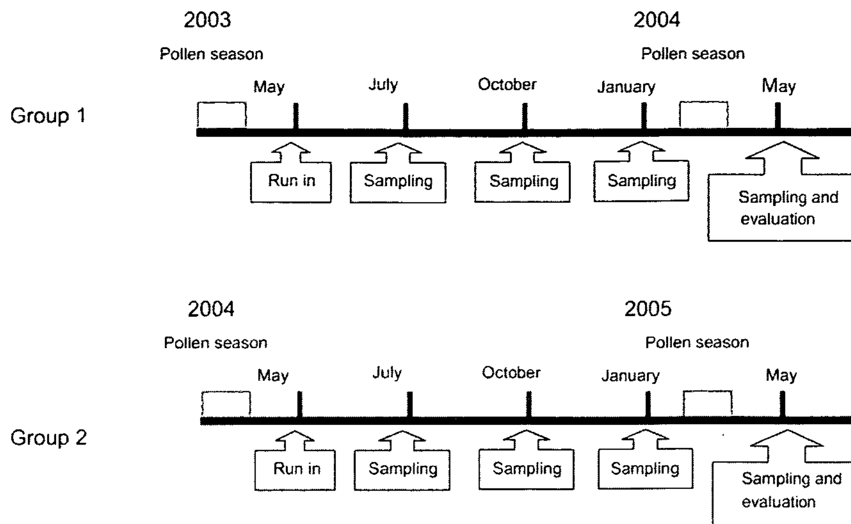
Table 1. Time schedule for examination



The Group 1 patients were recruited after the pollen season of 2003 and the Group 2 patients were recruited after pollen season of 2004. The blood sampling was performed every 3 months starting in July. The last blood sampling was performed after the pollen season of May, and the samples were analysed simultaneously.

cell determinants of Cry j 1 and Cry j 2, and the major Japanese cedar pollen allergens. Almost all the patient populations responded to this hybrid peptide, which is comparable to the response to Cry j 1 and Cry j 2. Moreover, because the seven peptides do not contain IgE-binding residues of *C. japonica* allergens, the recombinant peptide will not directly influence IgE-bearing cells such as mast cells, basophils and B cells [18]. The recombinant peptides for mite allergy were also prepared. This peptide comprised of the 14 CD4 T cell determinants of Der f 1 [19] and Der f 2 [20], and the major mite allergens. Almost all the patient populations responded to these peptides, which is comparable to the response to Der f 1 and Der f 2. Those peptides were Class II restricted and recognized Th cells only.

ELISPOT

The ELISPOT assay was performed according to the manufacturer's instructions. Briefly, the anti-human IL-4 or IFN-γ MoAbs were diluted to a concentration of 15 μg/mL in sterile, filtered (0.45 μm) PBS (pH 7.2), and 100 μL/well was added onto nitro-cellulose plates (Millititre, Millipore Corp., Bedford, MA, USA). The plates were incubated overnight at 4 °C and the unbound antibodies were washed with filtered PBS thereafter. After the last wash, the PBS was sucked through the membrane under vacuum (Millipore Corp.). One hundred microlitres of the pre-stimulated cell suspension was added to each well in duplicate, and the plates were incubated for 10 h at 37 °C. The cells were subsequently washed before adding 100 μL of the biotinylated MoAbs (1 μg/mL), and were then incubated for 2 h at room temperature. The plates were then washed and incubated for 90 min at room temperature with 100 μL of streptavidin alkaline phosphatase (Mabtech, Stockholm, Sweden) at a dilution of 1 : 1000. The unbound conjugate was removed by another series of rinsing before 100 μL of BCIP/NBT substrate solution (Bio-Rad, Richmond, CA, USA) was added, and the plates were incubated at room temperature until dark spots emerged (1 h). The colour development was stopped by repeated rinsing with tap water. After drying, the spots were captured photo-electrically and counted by a computed analysis to avoid any visual bias, using Auto Counter (ImmunoScan, CTL, Gmünd, Germany).

Pollen counts

The combined annual cedar and cypress pollen counts were measured using Durham pollen samplers.

Statistical analysis

Wilcoxon's paired rank sum tests were used to compare the mean values. A Friedman two-way ANOVA was used to

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analyse paired data. Peason's tests were used to determine the correlation coefficients.

Results

The cedar and cypress pollen counts determined using the Durham samplers were 470 cm/season in 2004, which was 1/20 of the average for the last 10 years. In 2005, we obtained 7852 cm/season, which was threefold higher than that of the average for the last 10 years. The seasonal symptoms were comparatively mild in 2004, and comparatively serious in 2005. The mean symptom-medication scores during the pollen season in 2004 and 2005 were 1.4 ± 2.1 and 2.8 ± 2.3 (mean $\pm$ SD), respectively.

Before the study, we compared the peptide-specific Th2 clone size of the patients with that of healthy controls in October, which is off pollen season. After stimulation with 10 nmol/L of cedar-specific peptides, the mean values of the IL-4 spot from the healthy controls and patients with cedar pollinosis that were obtained are shown in Fig. 1. Positive spots were obtained only in the samples from the pollinosis patients, but not from controls (A), although spots were equally obtained in the samples from both patients and controls when stimulated by Con A as a pan T cell stimulant (B). We also confirmed peptides for mite. The IL-4 spots were obtained only in the samples from the patients, but not from the controls (Fig. 2a), although spots were equally obtained in the samples from both patients and controls when stimulated by Con A as a pan T cell stimulant (Fig. 2b). Peptide-specific IL-4 spots were detected in all samples examined. Approximately 10–100 peptide-specific IL-4 spots were observed in the wells

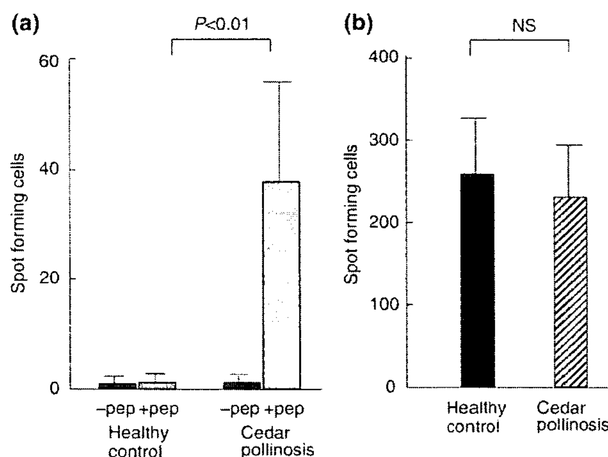

Fig. 1. The mean values of IL-4 spots in the samples from the healthy control subjects and patients with cedar pollinosis are shown. (a) 1×10^5 cells were stimulated by a peptide derived from Cry j 1 and Cry j 2. The spots were obtained only in the samples from the pollinosis patients, but not from the controls. (b) 1×10^4 cells were stimulated by Con A, a pan T cell stimulant. The spots were equally obtained in the samples from both the patients and the controls. NS, not significant.

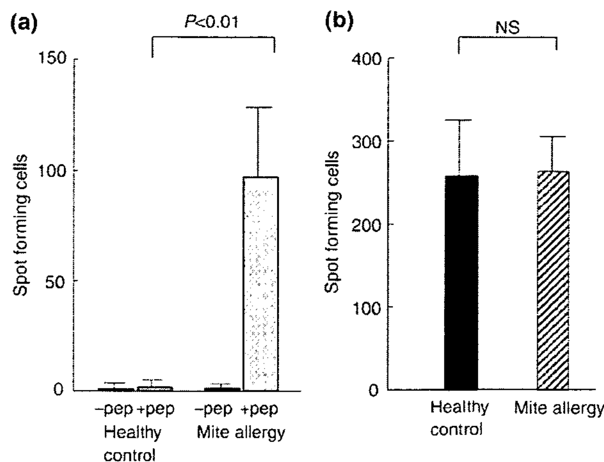

Fig. 2. The mean values of IL-4 spots in the samples from the healthy control subjects and patients with cedar pollinosis are shown. (a) 1×10^5 cells were stimulated by a peptide derived from Der f 1 and Der f 2. The spots were obtained only in the samples from the pollinosis patients, but not from the controls. (b) 1×10^4 cells were stimulated by Con A, a pan T cell stimulant. The spots were equally obtained in the samples from both the patients and the controls. NS, not significant.

incubated with 10^5 PBMC based on the ELISPOT assay. These spots were analysed in triplicate in each of the experiments and exhibited good reproducibility.

We carried out a depletion assay in advance with whole PBMC from three patients with Japanese cedar pollinosis using an antibody-conjugate magnetic bead kit (MACS system, Miltenyi Biotec GmbH, Tokyo, Japan). When CD4 was depleted, the spots of ELISPOT disappeared; however, after the depletion of CD8, the number of spots was equal to that with whole PBMC. The depletion of CD28 also caused the spots to disappear. These results show that the present ELISPOT assay using a hybrid peptide was CD4 restricted and the CD28 expression was indispensable for the detection of the spots (data not shown).

The seasonal changes in peptide-specific IL-4 spots are shown in Fig. 3. The IL-4 spots decreased after July, and were at their lowest in January before the onset of the cedar pollen season, which were almost 60% of those observed in July. The IL-4 spots increased during the cedar pollen season in 2004 despite the small amount of pollen (Fig. 3a). The same trend in seasonal changes was obtained during the 2005 season. The IL-4 spots decreased after July, and were at their lowest in January before the onset of the cedar pollen season, which were almost 60% of those observed in July (Fig. 3b). Interestingly, the clone size on May 2005 was 40% larger than that on July 2004. This phenomenon was not seen during the 2004 season.

The seasonal changes in the cedar-specific IgE levels in the serum of the patients are shown in Fig. 4. While the cedar-specific IgE decreased after the pollen season in 2003, it did not increase during the cedar pollen season in

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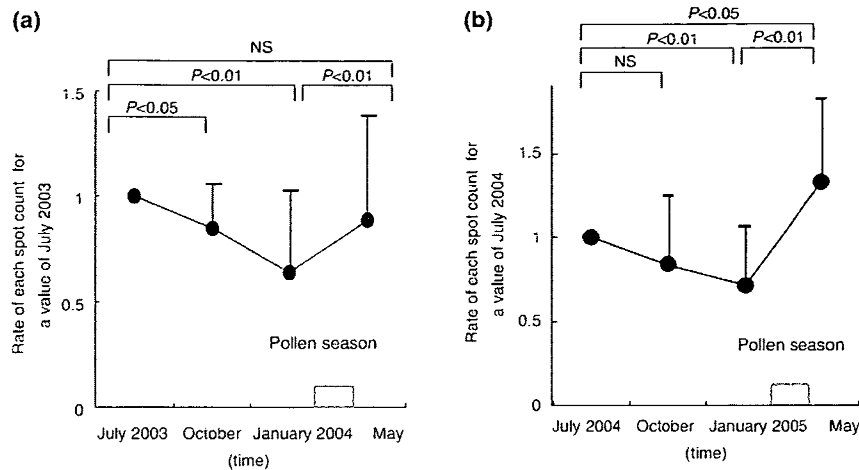

Fig. 3. The samples of each time-point were analysed simultaneously. The Cry j 1 and 2 peptide-specific IL-4-producing cells were counted using the ELISPOT technique. The rate of each spot count for the value in July is shown. (a) Twenty-three patients were enrolled during the 2004 season, which was characterized by very little pollen scattering. (b) Eighteen patients were enrolled during the 2005 season, which was characterized by massive pollen scattering. NS, not significant.

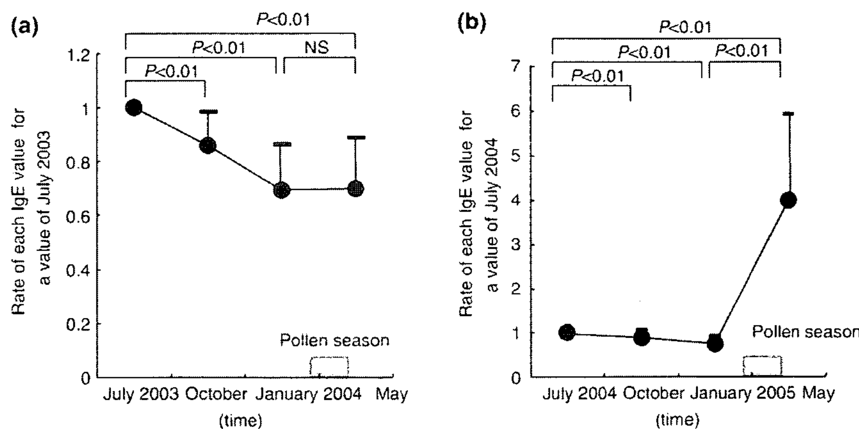

Fig. 4. The samples of each time-point were analysed simultaneously. Cedar-specific IgE were analysed using the RAST technique. The rate of each IgE for the value in July is shown. (a) Twenty-three patients were enrolled during the 2004 season, which was characterized by very little pollen scattering. (b) Eighteen patients were enrolled during the 2005 season, which was characterized by massive pollen scattering. NS, not significant.

2004 (Fig. 4a). The specific IL-4 spots decreased during the pollen season in some patients, and the cedar-specific IgE levels decreased during the pollen season in all patients. During the 2004–2005 season, the cedar-specific IgE levels decreased after the pollen season in 2004, but increased fivefold during the pollen season in 2005 (Fig. 4b). The number of cedar-specific IL-4 spots did not show a correlation with the cedar-specific IgE levels.

The yearly changes in the mite-specific IgE levels and Der f peptide-specific IL-4 spots were also examined in the samples from 22 patients with mite allergy. The yearly changes in mite-specific IgE and mite-specific memory Th2 clone size could not be clearly obtained (Fig. 5).

The patients with mite AR had persistent nasal symptoms all year around. Although the symptom scores varied among the patients as well as the seasons, the deviation was occasionally large; overall, no significant difference was observed. The pollinosis only demonstrated significant symptoms during the pollen dispersal season; however, the patients' nasal symptom scores during the pollen season were higher than those of the mite AR patients as shown in Fig. 6.

Discussion

We examined the Japanese cedar-specific IL-4-producing memory T cells in the peripheral blood of patients with

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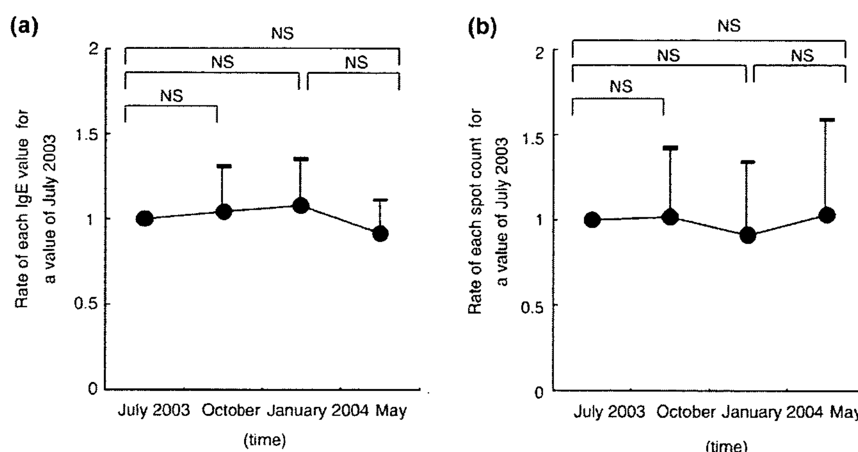

Fig. 5. The samples of each time-point were analysed simultaneously. Mite-specific IgE were analysed using the RAST technique (a). The Der f 1/2 peptide-specific IL-4-producing cells were counted using the ELISPOT technique (b). The rate of each value for the value in July is shown. NS, not significant.

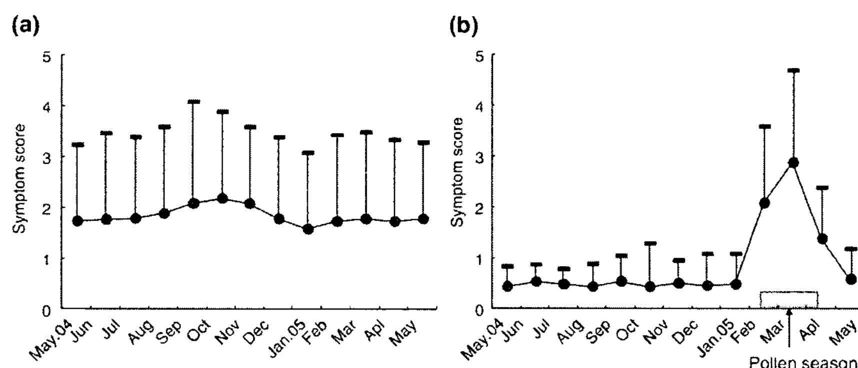

Fig. 6. The mean monthly symptom scores (mean+SD) from May 2004 to May 2005 are shown. (a) Mean nasal symptom scores of 22 patients with mite allergy. (b) Mean nasal symptom scores of 18 patients with Japanese cedar pollinosis.

Japanese cedar pollinosis by an ELISPOT assay using Japanese cedar-specific peptides. Although the number of cedar peptide-specific IL-4 T cells was low, the samples from all the patients examined had 10–100 spots/10^5 PBMC. In contrast, the healthy subjects had no positive IL-4 spot. These IL-4 T cells were thought to be cedar-specific Th2. The cedar peptide-specific IFN-γ T cells, which were thought to be cedar-specific Th1 cells, were detected in several patients. The cedar-specific peptides used in the ELISPOT assay were hybrid peptides that would only react with the T cells of those patients with Japanese cedar pollinosis, but not with the T cells of subjects not suffering from cedar pollinosis. Consequently, these findings could explain the low incidence and detection rates of cedar peptide-specific Th1 cells. The number of cedar peptide-specific IL-4 T cells (Th2 cells) did not correlate with the numbers of specific IFN-γ T cells (Th1 cells). More specifically, no relationship was observed between the cell types, and the cell numbers appeared to be independent of each other (although cedar-specific serum IgE levels did not correlate with the number of cedar-specific IL-4 T cells, the patients who had specific IFN-γ T cells had lower specific IgE levels than those without specific IFN-γ T cells).

IgE production is likely to be controlled by many factors. IFN-γ T cells inhibit IgE production independently of the number of specific IL-4 T cells [21, 22]. Similarly, pollen-specific IgE is known to exhibit seasonal changes [23, 24], thus increasing during the pollen season and decreasing during the off-season. However, the amounts of cedar and cypress pollen in 2004 were extraordinarily low and the pollen counts, which were less than 1/20 of the annual average for the last 10 years, did not contribute to the enhancement of IgE production in the patients enrolled in this study. Conversely, the cedar peptide-specific IL-4 T cells increased by 30% despite the low levels of pollen exposure. The levels of Th2 clone size increased to levels equivalent to that in July 2003.

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Although IL-4 is indispensable for IgE production, the cedar peptide-specific Th cells are thought to be very sensitive to exposure to cedar pollen.

In contrast, because the amount of pollen in 2005 was large, the cedar-specific IgE levels increased to five times after the pollen season. The specific Th2 clone size increased considerably after the pollen season, too, and the clone size increased by 70% after the pollen season. The levels of clone size increased to 1.4 times more than those observed in July 2004. A memory clone size might be affected by allergen exposition considerably. While cedar peptide-specific IL-4 Th2 cells decreased in number during the off-season, 8 months after the cessation of the pollen season, more than 60% of these memory Th2 cells were still found. Such annual drifting was absent in the perennial-allergic subjects. Therefore, pollinosis that undergoes exposition for a very limited period is different from mite perennial allergies.

Indeed, it is difficult to estimate the half-life of allergen-specific Th2. Generally, memory T cells are thought to have a long life-span, considering the duration of the vaccine effect of viral infections. Almost all reports of antigen-specific memory T cell have so far been about infectious diseases, while only a few reports of memory T cell are about type I allergy. The simple theory that an antigen creates a pool of long-lived antigen-specific memory T cells has been surprisingly difficult to prove and it is also still not completely accepted. The issue of longevity is much more controversial for $CD4^+$ than for $CD8^+$ T cells. Regarding $CD4^+$ memory T cells, lymphocytic choriomeningitis virus (LCMV)-specific $CD4^+$ memory T cells [25] and Sendai virus-specific $CD4^+$ memory T cells [26] at first disappeared dramatically, whereas virus-specific $CD8^+$ T cells were fairly stable in numbers over the same period. In humans, however, there have been several reports of vaccinia virus-specific $CD4^+$ memory T cells with a long life-span. These cells were found to decline with a half-life of approximately 10 years, conferring long-term protection [27]. The question remains, however, as to whether these memory Th cells are maintained in the absence of an antigen or by contact with a persistent antigen, bound in the immune complexes on follicular dendritic cells, or by contact with other environmental cross-reactive antigens. Based on our results on memory Th2 cells from cedar pollinosis in comparison with those from perennial mite allergy, the clone size maintenance requires antigen irritation of repetition and clonal mitosis by it. Therefore, the half-life of allergen-specific Th2 might be estimated to be less than 1 year.

The mechanism wherein a daughter Th2 cell discharges Th2 cytokines such as IL-4 in the same way after mitosis from the original Th2 has not been well understood. The opening and closing of chromatin helps to determine the cellular characteristics. It is believed that acetylation and methylation of the chromatin are very stable. Therefore, it is thought that the characteristics of a parent cell are transferred to the daughter cells after mitosis. However, a splitting enzyme for the acetylation and methylation has been discovered in a recent study, and it does not always seem to be stable [28]. It has recently been proposed that the opening and closing of chromatin is induced by an antigen, and that this is due to the Th cells' response [29, 30]. These changes in chromatin may therefore play an important role in retaining the memory functions of Th cells. Further studies need to be conducted on memory retention in order to clarify how these mechanisms could be applied to the development of an effective and fundamental solution for the treatment of allergic diseases.

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ORIGINAL ARTICLE

Preliminary Study on Japanese Cedar Pollinosis in an Artificial Exposure Chamber (OHIO Chamber)

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ABSTRACT

Background: A pollen exposure chamber (OHIO Chamber) was built in central Tokyo, Japan, in order to study seasonal allergic rhinitis (SAR). Since satisfactory outcomes were obtained from the controlled pollen exposure at the chamber, we conducted preliminary studies in volunteers with SAR.

Methods: Ten volunteers with SAR sensitive to Japanese cedar (JC) pollen were enrolled in this study. In order to investigate the intranasal and intraocular pollen number, volunteers were initially exposed to a low concentration of JC (2500 grains/m^3) for at most 1 hour in this chamber. Before and after the exposure, nasal cavities and eyes were washed with 100 ml and 25 ml of saline, respectively. Nasal and eye washing solutions were collected and the number of JC pollen was counted.

After 3 hours the volunteers were subsequently exposed to a moderate concentration of JC (4500 grains/m3) for 2 hours. Subjective nasal and ocular symptoms were recorded and the amount of nasal secretion was measured during the allergen exposure periods.

Results: During the initial exposure, all volunteers except one stayed in the chamber for 1 hour without any nasal or ocular symptoms. The number of pollen in the nose and eyes was 249.2 ± 120.9 and 13.6 ± 13.6 grains, respectively.

During the subsequent 2-hour exposure to JC pollen, nasal and ocular symptoms developed gradually in a time dependent manner in all the volunteers except one.

Conclusions: This is the first clinical study using Japanese cedar pollen under well-controlled conditions in the OHIO chamber in which the induction of allergic symptoms was observed. The OHIO chamber will be useful for studying allergic rhinitis in Japan.

KEY WORDS

allergen exposure, allergy symptoms, artificial exposure chamber, intranasal and intraocular pollen count, Japanese cedar pollen

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INTRODUCTION

Japanese cedar (JC) pollinosis is a seasonal allergy, which is unique to Japan, and the causative allergen (JC pollen) is dispersed usually between February and April although the pollen count varies every year. Over 16% of the Japanese population suffer from the allergy during this season[1] and an incredible amount of anti-allergy agent is used, resulting in an undermined QOL[2] and decline in labor productivity.[3] This fact indicates that JC pollinosis is a social and economical problem which cannot be ignored.

Allergen exposure tests in natural environments have been conducted as fundamental research on this kind of allergy and also to examine the effectiveness of anti-allergy agents.[4,5] Studies of this sort, however, are greatly influenced by natural factors such as the amount of pollen, the weather, the temperature, or

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the wind velocity. The biggest problem is that the tests can be done only during the pollen season.

Several allergen exposure units have been built in Europe and the US to evaluate the efficacy of drugs and they are operating safely and effectively.[6-8] A predefined amount of pollen can be dispersed under stable conditions in these chambers.

Despite the fact that so many Japanese are allergic to JC pollen, this kind of chamber was not available in Japan before 2004. To meet the increased needs for such facilities, the first pollen exposure units in Japan were developed in Wakayama[9] in the first half of 2005. We built the third allergen exposure chamber, an artificial exposure chamber (AEC; OHIO Chamber), in the center of Tokyo in September, 2005. This chamber not only keeps the temperature and humidity constant, but also automatically cleans and dries the inside of the chamber.

Due to the lack of AECs in Japan, little fundamental data concerning Japanese cedar pollinosis were available up to this point. Since satisfactory outcomes were obtained from the controlled exposure at the OHIO Chamber, we were able to conduct preliminary studies on mildly symptomatic patients, in which we examined the amount of intranasal and intraocular pollen grains and the development of symptoms.

METHODS

SUBJECTS

The subjects of our study were mildly symptomatic adult patients. The inclusion criteria were as follows:

- Subjects must have at least a 2-year history of allergic symptoms during the pollen season, such as sneezing, nasal discharge, nasal obstruction, and itchy eyes.
- Subjects must also have had blood tests within 1.5 years showing positive RAST scores (≥Class 2) for JC pollen and negative RAST scores (≤Class 1) for house dust mite.

The exclusion criteria were as follows:

- Subjects with nasal obstruction attributable to a polyp or deformity of the nose.
- Subjects with acute upper respiratory infection(s) such as acute sinusitis, acute pharyngitis, and acute upper respiratory inflammation.
- Subjects with uncontrolled asthma, diabetes, high blood pressure, or eye diseases (glaucoma/cataract).
- Subjects who used anti-allergy agent(s) within a week before the start of this study.
- Pregnant women or women who trying to become pregnant.

The study was conducted in accordance with GCP Guidelines and the Declaration of Helsinki. The study was conducted on December 23 (2 months ahead of the start of the pollen season) after having been reviewed and approved by the ethics committee of Shinanozaka Clinic. Informed consent was obtained from all the subjects prior to study entry.

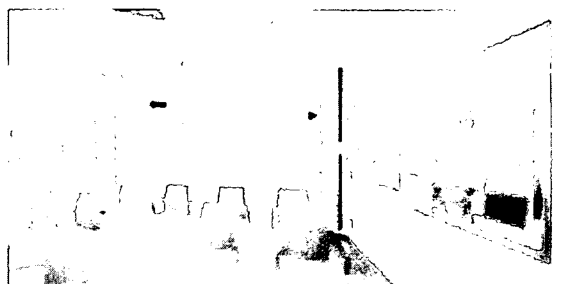
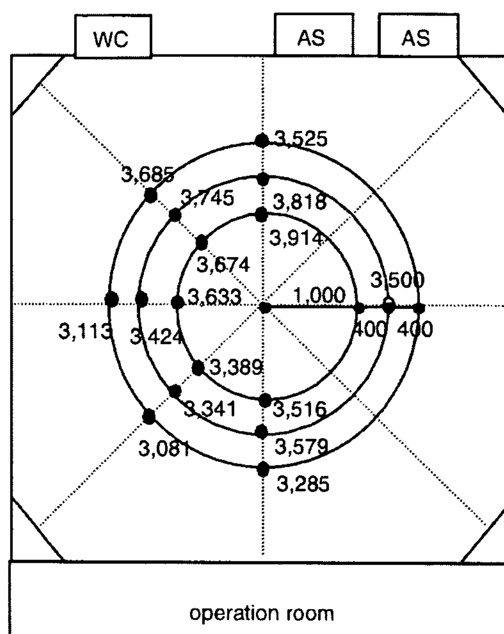


Fig. 1 Appearance of the artificial exposure chamber (OHIO Chamber). Pollen-diffuser in the center (arrow). Blower module in the corner of the room (arrow head)



Target concentration: 3500 grains/m^3

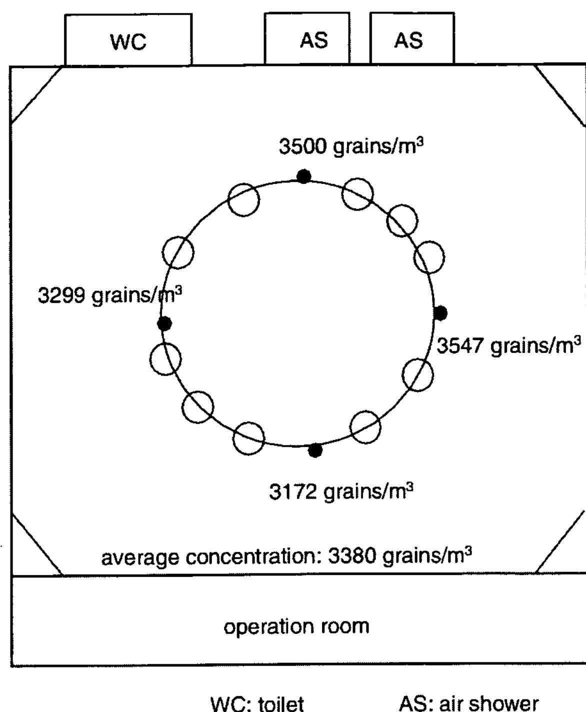
WC: toilet AS: air shower

Fig. 2 Spatial concentration distributions of pollen in the OHIO Chamber at a height of 1.15 m at the target concentration of 3500 grains/m^3. The average concentration of pollen was 3500 ± 419 grains/m^3.

OHIO CHAMBER

The OHIO Chamber was installed in Samoncho Clinic in Yotsuya, the center of Tokyo. Its square measure is 25 m^2, the height 2.5 m, and the capacity 10 subjects (Fig. 1).

Compressed air transfers pollen grains from an outside dust feeder into the operation room where it is mixed with conditioned air inside the pollen diffuser after which the mixed air jets out upward from the diffuser. The diffuser and the blower modules in



WC: toilet AS: air shower

Fig. 3 The pollen concentration at a height of 1.15 m at the four points (●) when the ten volunteers (○) were present in the OHIO chamber. The average concentration was 3380 grains/m^3 and uniform distribution of pollen was achieved.

four corners help the pollen grains get distributed evenly. The concentration of pollen grains in the room is measured by a particle counter equipped with a semiconductor laser (KC-20; Rion Co, Tokyo, Japan). This counter can absorb up to 30 L of air per minute and detects particles sized 10–100 μm.

Spatial distributions of pollen in the unmanned and manned chamber at a height of 1.15 m were measured. The pollen concentration distribution was within a range of ± 12% of the target value of 3500 grains/m^3 in the unmanned chamber (Fig. 2). The pollen number at each of four points and the average pollen concentration are shown in Figure 3 when 10 subjects were present in the chamber.

The pollen count in this room is stable between 2500 and 120000 grains/m^3 (data not shown). Also, the temperature inside the chamber was set at 22 ± 0.2℃ and the humidity was set about 20–55%. The pollen count, temperature, and humidity were monitored every 3 minutes and recorded accordingly.

Before entering the room, subjects were instructed to wear protection gowns so that the pollen grains were not attached to their hair or clothes. When entering or exiting, they also went through an air shower so as to avoid pollen grain contamination outside of the room. During the test, subjects were assigned to sit on chairs in designated areas of the chamber and in order to ensure the subjects' safety, their behavior was observed closely by staff and physicians situated in the operating room.

Table 1 Backgrounds of the subjects.

Subject No.	Sex	Age	RAST score
1	female	37	3
2	female	35	5
3	male	34	4
4	male	45	3
5	male	36	2
6	female	34	4
7	male	41	2
8	female	32	2
9	female	43	3
10	male	48	2

Five men and five women with mild symptoms were enrolled in this study.
This table shows their age and antibody titers (RAST scores) against cedar pollen.

STUDY DESIGN

This study consisted of two parts. In the first part, subjects were exposed to low-concentrated JC pollen (2500 grains/m^3) in the chamber for up to an hour. They were allowed to exit the room if allergic symptoms developed (either in the nose or in the eyes). Immediately after exiting the room, all the subjects went through an intranasal and intraocular irrigation process using a syringe (Nasaline®; Entpro, Sweden), washing the nasal cavities with 100 ml of saline and the eyes with 25 ml of saline. Then the washing solution from each subject was collected to investigate the number of pollen grains in it.

The subjects were subsequently exposed to moderate-concentrated JC pollen (4500 grains/m^3) for 2 hours. Each subject recorded their symptoms (such as sneezing, nasal discharge, nasal obstruction, itchy nose, itchy eyes, and tears) into computers at certain points (after 15, 30, 60, 90, and 120 minutes from the start of exposure).

The symptoms were classified as follows: 1. none, 2. mild, 3. moderate, 4. severe, and 5. very severe. We collected tissue papers with which subjects had blown their noses and measured them by weight. The weight difference between tissue papers before and after use was considered to be the weight of nasal secretion.

The amount of intranasal and intraocular pollen was determined by the following methods. We added 1.25 *g* of Safranine-O (Wako Jyunnyaku Kogyo Inc. Tokyo) to 50 ml of saline and 50 ml of ethanol to create stain solutions, and 5 ml of this stain solution was added to the nasal or ocular lavage fluid. Furthermore, we added 20 drops of Proteinase K (Dako Cy-