and more than 70% of patients who are allergic to cedar pollen also develop cypress pollinosis [5, 6]. Around the Tokyo region, the cedar pollen season usually starts in the middle of February and is followed by the cypress pollen season which lasts until the beginning of May. Consequently, many patients with cedar pollinosis suffer from heavy pollen exposure for almost 12 weeks. In addition, Japanese cedar and cypress pollen can travel more than 100 km from the source, thereby raining down large amounts of pollen on other large cities. This situation is considerably different from that experienced in other countries where the most common allergens are grass pollens and ragweed, which generally travel distances of several hundred meters, and the pollen season lasts for less than 6 weeks [7]; however, it is similar to Northern European countries and North America where birch and other tree pollens are the major contributors.

Subcutaneous allergen-specific immunotherapy (SCIT) has been evaluated and shown to be an effective approach to change the course of allergic rhinitis [8–13], including Japanese cedar pollinosis [14]. However, an alternative method of administration is still required because the SCIT approach has been associated with the risk, albeit very low, of anaphylactic shock [15] and the inconvenience of frequent visits to the physician's office.

A recent review of randomized controlled studies of sublingual immunotherapy (SLIT) conducted outside Japan has strongly suggested its efficacy against a variety of allergens [16–21]. SLIT could be an attractive approach for Japanese cedar pollinosis if efficacy, safety, mechanisms and effective biomarkers can be clearly established.

The present placebo-controlled randomized studies were designed to determine the effects of SLIT on Japanese cedar pollinosis employing recombinant hybrid peptides consisting of 7 HLA class 2 restricted T cell epitopes of Cry J, the major allergen of Japanese cedar pollen [22].

Methods

Subjects

The study population consisted of 67 patients (33 males and 34 females), ranging in age from 20 to 37 years, who were otherwise healthy, but had a clinical history of Japanese cedar pollinosis for at least the last 3 consecutive cedar pollen seasons. The subjects lived in and around the city of Chiba, where a similar amount of pollen spread would be expected. The diagnosis of cedar pollinosis was based on clinical history, positive allergen-specific skin tests (wheal diameter ≥10 mm) to a standardized cedar pollen extract (Torii Pharmaceutical Co. Ltd., Tokyo, Japan) and serum cedar pollen-specific IgE levels of ≥score 2 by the CAP radioallergosorbent test (CAP-RAST; SRL, Tokyo, Japan). The exclusion criteria included a history of severe asthma, use of antiallergic drugs within 4 weeks and a prior history of any allergen-specific immunotherapy, including therapy for cedar pollen. Pregnant women or those at risk of pregnancy were also excluded. The study was conducted at the Chiba University Hospital and the protocol was approved by the Ethics Committee of Chiba University; written informed consent was obtained from each of the patients prior to participation in this study.

Japanese Cedar Pollen Extracts

Standardized Japanese cedar pollen extracts (Torii Pharmaceutical Co. Ltd.) were used [23]. The extract [1,000 Japanese Allergy Units (JAU)/ml] contained 1.5 μg of Cry j 1, which is the major allergen of Japanese cedar pollen. The amount of Cry j 1 was quantitated by an enzyme-linked immunosorbent assay, as reported previously [24].

Study Protocol

The study was placebo controlled and single blinded. The enrolled subjects were randomly divided into 2 groups with a ratio of 2:1 according to the table of random numbers by the Department of Pharmacy at the Chiba University Hospital. A controller who was not directly involved in this study was responsible for group allocation. The patients were divided randomly into the active (treatment) and placebo groups. A group allocation number was given to each patient. To prevent the leakage of information, this number was closely guarded jointly by the controller and a member of the ethical committee who was also not directly involved in the study, until accessed with the key after the completion of the study. The active group consisted of 43 patients who received the pollen extract and the placebo group consisted of 24 patients who received the placebo (inactive) for sublingual administration by the spit method (table 1). The sample size was determined based on previous similar studies [25]. The induction/buildup phase was 1 month, with the administration of an increasing daily number of the extract drops at 3 concentrations. The patients received 1 ml of 1,000 JAU extract or placebo once weekly as shown in table 2. Although the safety of the daily administration of SLIT has been reported recently, the weekly administration was chosen in this study in order to further reduce the possibility of any serious adverse events. No study of SLIT for Japanese cedar pollinosis has been reported to date. However, the development of asthma attacks by exposure to pollen has been observed in some patients [14]. The maintenance dose of the antigen in the present SLIT studies was about 100 times higher than that routinely used in SCIT. Administration was started at the beginning of October 2005 and ended at the end of April 2006. The patients carefully completed a pollen diary regarding their nasal symptoms and the usage of rescue drugs (such as antihistamines). Data were collected and analyzed at the Department of Clinical Testing of the Chiba University Hospital.

The nasal symptoms were evaluated on a scale from 0 to 4 in accordance with the *Practical Guidelines for the Treatment of Allergic Rhinitis, Japan* [26], as follows: 0 = no sensation; 1 = mild; 2 = moderate; 3 = severe; 4 = extremely severe. Daily episodes of sneezing and nose blowing were rated 0–4, as follows: 0 = none; 1 = 1–5 episodes; 2 = 6–10 episodes; 3 = 11–20 episodes; 4 = more than 20 episodes. The medications were also recorded according

to drug characteristics and duration of usage, according to the guidelines as follows: antihistamines, mast cell stabilizers and vasoconstrictors were listed as 1, topical ocular or nasal steroids as 2.

Immunoglobulin Assay

Serum Cry j 1-specific IgG4 antibodies were measured using microtiter plates coated with 100 ng/well of Cry j 1 which was purified as reported previously [27]. Allergen-coated wells with serum samples (diluted 1:50) were incubated for 2 h at 37°C, and then washed with PBS. The plates were incubated with 100 μl of biotinylated monoclonal anti-IgG4 antibody (BD Pharmingen; 500 ng/ml) for 1 h at 37°C, and then overnight at 4°C. After washing, the plates were incubated with 100 μl of streptavidin-γ-D-galactosidase conjugate (Roche Diagnostics) at 1:2,000 dilution for 1 h at 37°C, and washed. Finally, 100 μl of 5 mM o-nitrophenyl-β-D-galactopyranoside was added to the wells and incubated for 1 h at 37°C. After the enzyme reaction was stopped with 100 μl of 0.1 M Na_2CO_3, the absorbance at 415 nm was read using a microplate reader.

The specific IgG4 antibody levels were calculated from control curves with serial dilutions of a reference serum pool, which was prepared from 5 sera with high levels of Cry j 1-specific IgG antibody. The IgG4 antibody levels in the reference pool serum were arbitrarily assigned to be 100 U/ml.

Analysis of Th Cytokines and Cell Clones

Peripheral blood mononuclear cells (PBMCs) were obtained by the Ficoll-Hypaque method and stored at –80°C until analysis, using a cell banker (Nippon Zenyaku Kogyo Co. Ltd., Fukushima, Japan).

Th1/Th2 cytokine profiles were analyzed using FACS analysis. PBMCs (5×10^5) were stimulated with PMA and ionomycin for 4 h in the presence of 2 μM monensin, which inhibited the secretion of protein produced de novo. The cells were stained with anti-CD4 antibody for 15 min on ice. After washing with PBS, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized with 0.5% Triton X-100 for 10 min on ice. After blocking with 3% bovine serum albumin for 10 min, the cells were incubated on ice for 30 min with anti-IFN-γ labeled with fluorescein isothiocyanate and anti-IL-4 labeled with phycoerythrin. A flow-cytometric analysis was performed on FACS Calibur (Becton Dickinson, Irvine, Calif., USA). The antibodies for FACS analysis were purchased from BD Bioscience (San Diego, Calif., USA).

The Cry j-specific Th2 clone sizes were determined by an ELISPOT assay using the recombinant hybrid peptide. The hybrid peptide comprised the 7 CD4 T cell determinants of Cry j 1 and Cry j 2, the major Japanese cedar pollen allergens [22]. Almost the entire patient population with Japanese cedar pollinosis respond to this hybrid peptide and the responses are comparable to the individual responses to Cry j 1 and Cry j 2 [22]. The monoclonal antibodies used in the ELISPOT analysis were obtained from Mabtech (Stockholm, Sweden). The anti-human IL-4 or IL-5 monoclonal antibodies were diluted to a concentration of 15 μg/ml in sterile, filtered (0.45 μm) PBS (pH 7.2), and 100 μl per well were added onto nitro-cellulose plates (Millititer; Millipore Corp., Bedford, Mass., USA). The plates were incubated overnight at 4°C and the unbound antibodies were washed with filtered PBS thereafter. After the last wash, PBS was sucked through the membrane under vacuum (Millipore). One hundred microliters of AIM-V medium with or without 20 μM hybrid peptide was added to 5×10^5 cells per well, and the plates were incubated for 10 h at 37°C. All assays were done in duplicate. The cells were subsequently washed before adding 100 μl of the biotinylated monoclonal antibodies (1 μg/ml), and incubated for 2 h at room temperature.

The plates were washed and incubated for 90 min at room temperature with 100 μl of streptavidin alkaline phosphatase (Mabtech) at a dilution of 1:1,000. The unbound conjugate was removed by another series of rinsing before 100 μl of BCIP/NBT substrate solution (Bio-Rad, Richmond, Calif., USA) was added and the plates were incubated at room temperature until dark

Table 1. Baseline characteristics of the patients

	Treatment group (n = 43)	Placebo group (n = 24)
Mean age, years[1]	26.8 ± 5.4	26.4 ± 5.9
Female sex	21 (48.8)	13 (54.2)
Mean duration of cedar pollinosis, years	8.7	9.1
Type of allergic rhinitis		
Cedar pollinosis with perennial	7 (16.3)	3 (17.5)
Cedar pollinosis with other pollinosis	5 (11.6)	4 (16.7)
Cedar pollinosis only	31 (70.5)	17 (70.8)
Additional allergic history		
History of asthma symptoms	2 (4.6)	0
Current asthma symptoms	0	0
History of allergic conjunctivitis	40 (93.0)	19 (75.0)
Cedar pollen RAST score[1]	4.18 ± 1.01	4.14 ± 0.92
Peak of daily total nasal symptoms score in the last cedar pollen season	4.8	4.5

Figures in parentheses are percentages.
[1] Data are means ± SD.

Table 2. Dose and dosing frequency

	Week 1 20 JAU	Week 2 200 JAU	Week 3 2,000 JAU	Week 4 2,000 JAU
Day 1	0.2 ml	0.2 ml	0.2 ml	1.0 ml
Day 2	0.4 ml	0.4 ml	0.4 ml	
Day 3	0.6 ml	0.6 ml	0.6 ml	
Day 4	0.8 ml	0.8 ml	0.8 ml	
Day 5	1.0 ml	1.0 ml	1.0 ml	
Day 6				
Day 7				

The induction phase with an increasing number of extract drops over 5 days a week at 3 concentrations for 3 weeks and the maintenance phase (week 4) with 1 ml of 1,000 JAU extracts once weekly are shown.

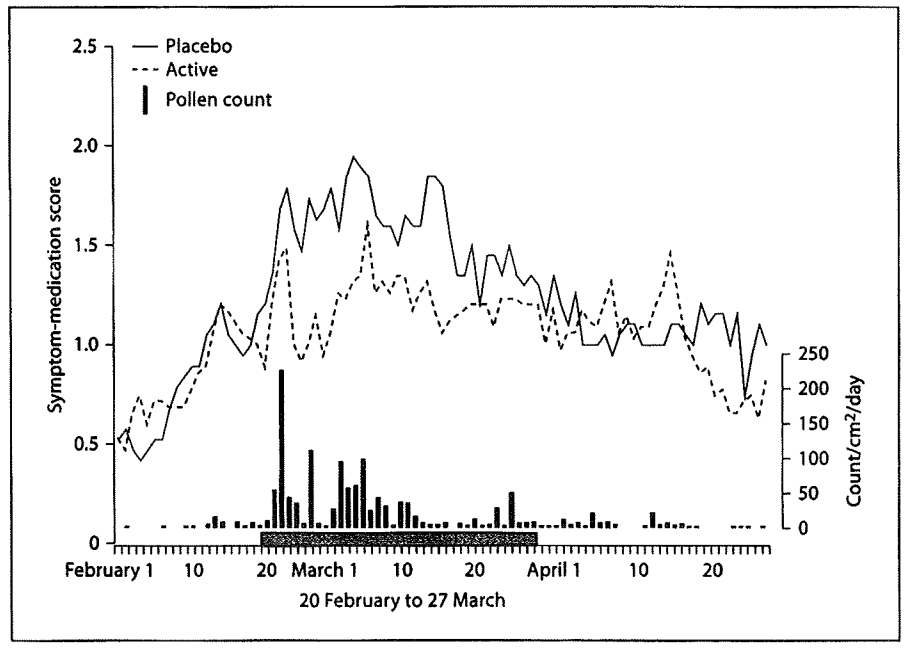
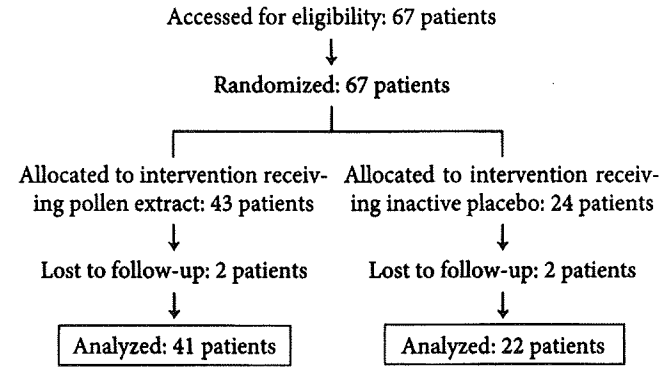


Fig. 1. Daily combined Japanese cedar and cypress pollen counts in 2006 in Chiba measured by the Durham pollen sampler and symptom-medication score (mean values) of patients during the pollen season.

Table 3. Study participation



spots emerged (1 h). The color development was stopped by repeated rinsing with tap water. After drying, the spots were captured photoelectrically and counted by a computed analysis to avoid any visual bias, using an auto counter (ImmunoScan; CTL, Cleveland, Ohio, USA).

Statistical Analysis

After completion of the study, the clinical and laboratory data were analyzed by a biostatistician who was not involved in carrying out the clinical trial. After completing the analysis, the allocation identification numbers for the active and placebo groups were accessed with a key. The Mann-Whitney U test was performed to compare symptom scores as well as symptom-medication scores between placebo and active groups. The Wilcoxon signed rank test was used for paired comparisons of the Cry j 1 specific IgG4 levels before and after SLIT. All statistical analysis was performed using the GraphPad Prism software, version 4.

During the statistical calculations in the present studies, the β error was defined as 0.2, power was 80% and the α error was defined as 0.05. Values of $p < 0.05$ were considered statistically significant.

Results

Four patients were withdrawn from the study for personal reasons, but not due to any adverse effects. All other subjects exhibited full compliance with the study protocol. As a result, 63 patients (41 patients from the active group and 22 patients from the placebo group) were analyzed further for effectiveness of SLIT (table 3).

Adverse Effects

Fifteen adverse effects were reported during the treatment. Of these, 13 subjects were in the active treatment group and 2 in the placebo group. Two patients in the active group complained of mild urticaria of the face or breast. The remaining subjects exhibited mild oral pruritus or oral pain (Common Terminology Criteria for Adverse Event grade 1). All adverse effects were transient and resolved spontaneously. No intervention was necessary.

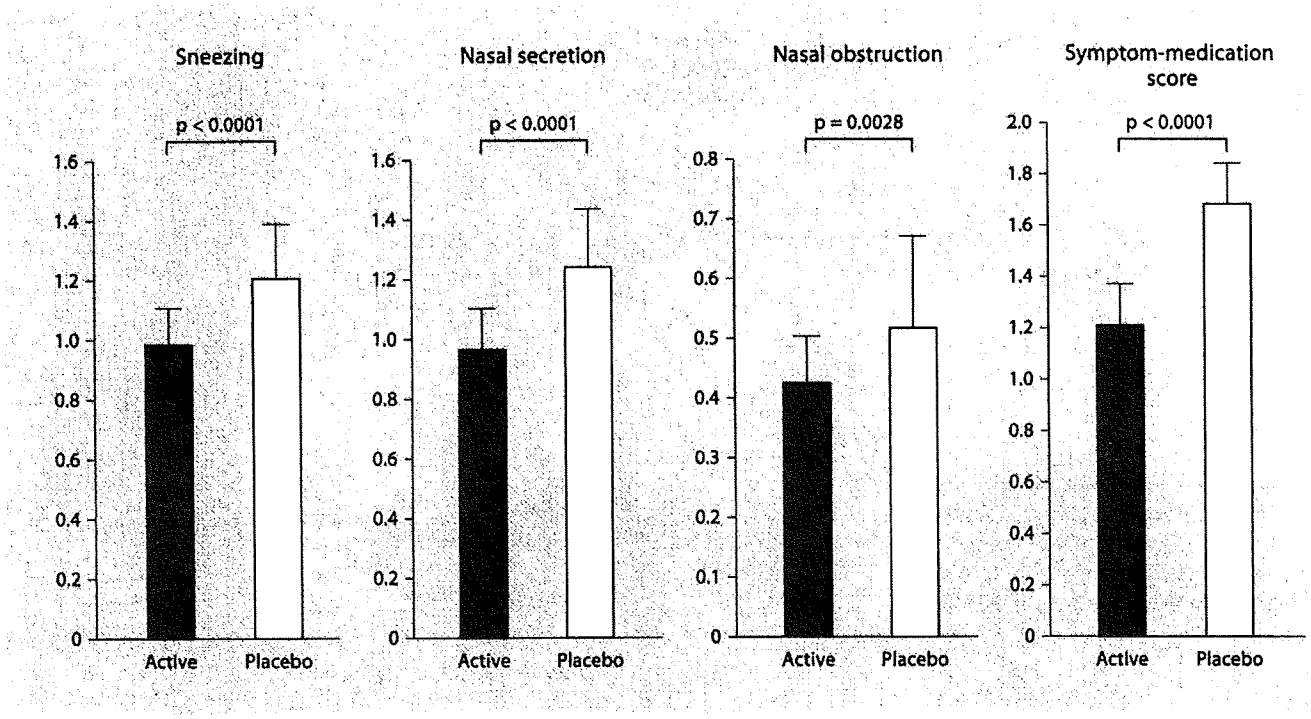


Fig. 2. Average symptom scores of sneezing, nasal secretion, nasal obstruction and symptom-medication score during the high pollen season, from 20 February to 27 March 2006. The average score of the active group was significantly lower than that of the placebo group.

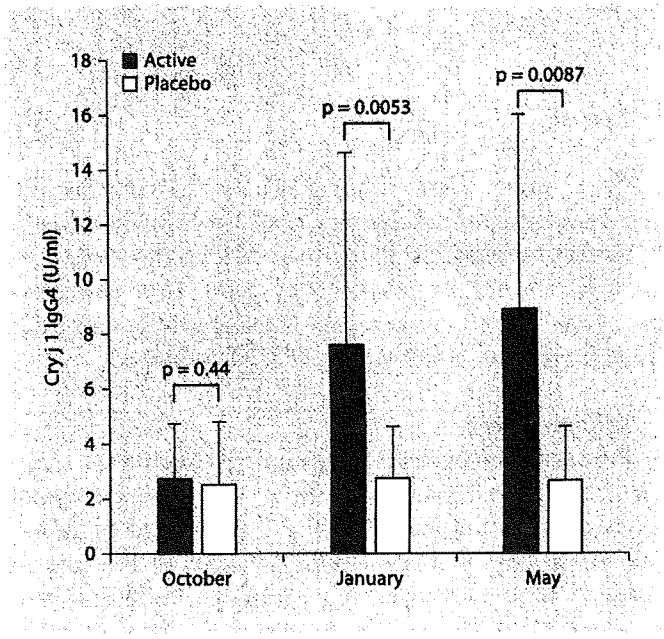


Fig. 3. Levels of serum Cry j 1-specific IgG4 before/after pollen dispersal. Specific IgG4 significantly increased in the active group but not in the placebo group and a significant difference was observed between the groups ($p < 0.05$).

Pollen Counts

In 2006, the Japanese cedar pollen season started in the middle of February and was followed by cypress pollen, which continued until the end of April. The duration of the pollen season extended from 20 February to 27 March. The combined annual amount of cedar and cypress pollen was 1,154/cm^2 according to the Durham pollen sampler in Chiba.

Symptoms

The nasal symptoms and medication scores during the pollen season are shown in figures 1 and 2. The temporal profiles of nasal symptoms and medication scores were in general similar in the active and placebo groups and reflected the pollen counts in the community. However, the scores were lower in the active (treatment) group, especially during the peak of the pollen season as shown in figure 1.

The symptom scores for sneezing, nasal secretion volume, degree of nasal obstruction and medication scores were significantly higher in the placebo group compared to the active (treatment) group ($p < 0.01$) during the peak of the pollen season as shown in figure 2.

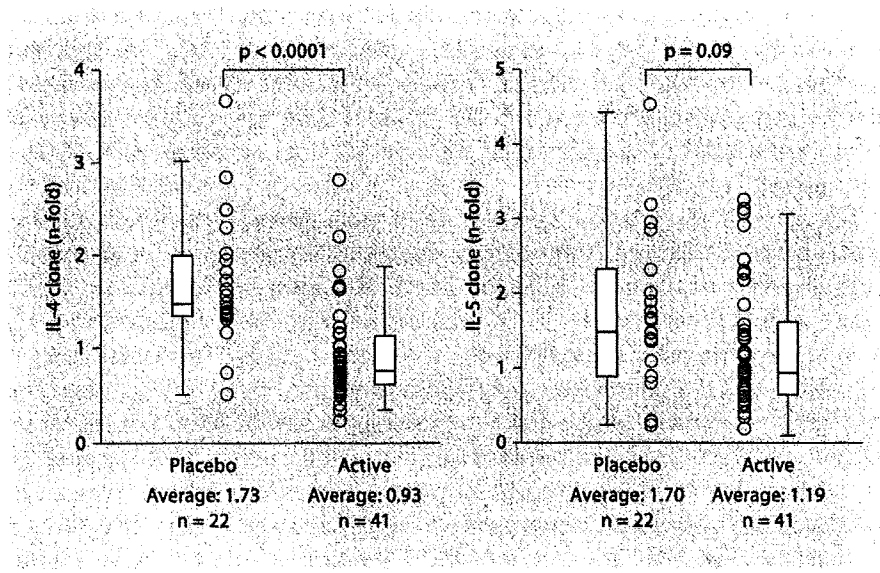


Fig. 4. Relative change of Cry j-specific IL-4 and IL-5 clone sizes in May, after the pollen season, compared with those in January before the season.

Serum Immunoglobulin

There were no significant differences in the 2 study groups for the Japanese cedar pollen-specific IgE and IgG4 levels in the serum samples collected in October, just before SLIT was initiated. On the other hand, after the initiation of immunotherapy, Cry j-specific IgG4 levels exhibited a significant increase in the active group in January before the pollen season. Significantly higher levels of specific IgG4 were observed in the active group for at least 4 months after the initiation of the immunotherapy as shown in figure 3. No significant effects of immunotherapy were detected for the levels of specific IgE (data not shown). No changes in the specific IgE levels were observed relative to the cedar pollen dispersion between January and May. The levels of specific IgG4 also did not exhibit any change after pollen exposure in both the active and placebo groups (fig. 4) and the levels did not correlate with the nasal symptom scores (data not shown).

Th1/Th2 Cytokine Profiles

The number of Th1/Th2 cells in the peripheral blood CD4 T cells did not change significantly and no significant difference was observed between the 2 groups during the study period (data not shown).

Cry j-Specific Th2 Clone Sizes

The number of Cry j-specific IL-4 and IL-5 spots showed a strong correlation. Although the number of spots was similar between the active and placebo groups before the pollen season, a significant increase in IL-4 spots was observed only in the placebo group after the pollen season. The increase in IL-4 clone size during the pollen season in the active and placebo groups was 1.71 ± 0.71 and 0.70 ± 0.52 (mean ± SD), respectively ($p < 0.0001$). On the other hand, the increase in IL-5 clone size between the active and placebo groups was not significant, the power ($1 - \beta$ error) was 0.58 ($p = 0.09$) as shown in figure 4.

Discussion

Although the use of SCIT has been found to be safe for immunotherapy for a variety of pollen allergies, the practical inconvenience associated with its use prompted this study to explore alternative routes of administration. A recent review of randomized controlled studies of SLIT has suggested both its efficacy and safety [16–21]. Although SLIT for Japanese cedar pollinosis is an attractive alternative route, no randomized controlled studies have been carried out to date. The observations of particular importance reported here have shown that the use of SLIT significantly increased the levels of pollen-specific IgG and downregulated the size of pollen-specific Th2 lymphocyte subset clones.

In order to avoid adverse effects, such as local pain and swelling associated with injection and possible anaphylactic reactions, a dose of 40 JAU/month as a maintenance dose has generally been utilized in SCIT for Japanese pol-

linosis. In this study 1,000 JAU/week (4,000 JAU/month) was used as a maintenance dose in SLIT, which was 100 times more than that used in SCIT. The choice for such a dose was somewhat arbitrary and the optimal dose required for effective and safe use of SLIT remains to be determined.

The combined Japanese cedar and cypress pollen counts generally exceed 3,000/cm^2, as measured by the Durham pollen sampler in Chiba and Tokyo. However, in 2006 the pollen counts were 1,154/cm^2, which was one third of the average for the last 5 years. In Japan, the pollen counts and the counts/cm^2 are usually measured by the Durham samplers, which utilize a gravimetric method that is different from the Burkard sampler, a volumetric method that is widely used in European countries. Direct comparison of the counts by the 2 methods can be difficult, because the ratio between the 2 methods depends on the local meteorological conditions and the types of pollen. When these methods were compared in 2005, the counts obtained by the Burkard sampler were about 12 times higher than those obtained by the Durham sampler [28].

During SLIT, no adverse effects greater than Common Terminology Criteria for Adverse Event grade 3 were observed. Three months after SLIT, serum Cry j 1-specific IgG4 was elevated in the active group. However, the specific IgE levels were not significantly different between the groups.

Several previous studies employing SLIT have observed an increase in allergen-specific IgG4 levels and specific IgG4/IgE ratios in the serum [29, 30]. However, the precise role of increased IgG4 in the effectiveness and outcome of such immunotherapy remains to be determined. Lima et al. [31] reported that the IgG levels correlated with the clinical efficacy as a blocking antibody, but other studies have failed to demonstrate such a correlation [32]. The increased levels of Cry j 1-specific IgG4 antibody in this study indicate that SLIT can induce specific antibody responses. However, the role of IgG4 antibody in the mechanisms of clinical effectiveness remains to be defined. No relationship between the IgG4 responses and the clinical efficacy was observed in this study.

In the present studies, the use of SLIT was associated with milder clinical symptoms and lower medication scores during the pollen season and a significant reduction in each symptom was observed. As pointed out earlier, the doses in this SLIT study were much higher than those generally used in SCIT. However, in SLIT with other allergens, the clinical efficacy has been shown to be allergen dose dependant [33]. Although the swallow-SLIT method is currently widely used, we selected the spit-SLIT method to further reduce the possibility of adverse effects, since no SLIT trials with Japanese cedar pollinosis have been carried out to date. Further studies will be needed to assess the dose responses, temporal intervals and vehicles of administration to obtain optimal effectiveness with SLIT.

Cedar pollen-specific IgE and IgG4 did not increase significantly with pollen exposure, which might be explained in part by the relatively small amount of pollen dispersal observed during these studies.

The total number of Th2 cells in the peripheral blood did not increase during the pollen season and Th1/Th2 cytokine profiles did not change throughout the study in either group. However, the profiles of the allergen-specific Th cells were quite different. The patients with cedar pollinosis are thought to have cedar pollen-specific memory Th cell clones and the treatment is aimed at diminishing the size of Th2 clones. Since the Th cell response is restricted in MHC class 2, it is necessary to use a class 2 restrictive T cell epitope to measure the reaction of T cell clones in response to the allergen. Japanese cedar-specific IL-4 and IL-5 producing memory T cell in the peripheral blood were examined by an ELISPOT assay using Japanese cedar pollen-specific peptides. Although the number of cedar peptide IL-4 and IL-5 T cells was low, all patients exhibited specific spots, ranging from 5 to 100 spots/10^5 PBMCs. The number of cedar pollen-specific Th2 cells did not correlate with the cedar pollen-specific serum IgE nor IgG4 levels. This may be related to the possibility that IgE and IgG4 synthesis is controlled by many other factors, including Th1 cells and memory B cells.

The size of the cedar pollen-specific Th2 cell clones was not different between the active and the placebo groups before the pollen season. Interestingly, these Cry j-specific Th2 clone sizes were increased about 1.7-fold during the cedar pollen season by pollen exposure in the placebo group. However, this increase was not observed in the active group and SLIT suppressed the increase in specific Th2 clone sizes. The change in the clone size did not correlate with the levels of allergen-specific IgG4 antibody.

Several recent studies have explored the significance of regulatory T cells in allergic and autoimmune disorders [34–37]. The suppression of allergen-specific Th2 clones observed in this study may be a reflection of such regulatory T cells, although the precise contribution of different T cell subsets remains to be examined.

In summary, this study has demonstrated that SLIT for Japanese cedar pollinosis was safe and is associated with increased cedar pollen-specific IgG4. Such therapy also inhibited the increase in specific Th2 lymphocyte clone sizes induced by the exposure to cedar pollen. It is also suggested that the use of SLIT appears to be an acceptable alternative to SCIT for Japanese cedar pollinosis.

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ORIGINAL ARTICLE

Association study of the *C3* gene with adult and childhood asthma

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Abstract Bronchial asthma (BA) is a multifactorial disorder, the development of which is affected by both environmental and genetic factors. The complement system plays an important role in immunological response against invading microorganisms. It has been shown that complement-C3-deficient mice have reduced inflammation of asthmatic airways. Previously, we reported the association of four single nuclear proteins (SNPs) in the exons of the *C3* gene with childhood and adult BA. The *C3* gene, however, is a large gene, and functional SNPs associated with susceptibility to BA have not yet been identified. We analyzed 26 SNPs in the *C3* gene and its promoter region to narrow down the regions showing association with childhood and adult BA. Childhood and adult atopic BA patients and healthy child and adult controls were recruited from urban cities in Japan and genotyped. In SNP analysis, an SNP (SNP24, rs11569562) located in intron 31 of the *C3* gene was associated with adult BA [corrected P (P_{cor}) = 0.030]. In linkage disequilibrium (LD) block 4 spanning exons 24–41, the frequency of the CCC haplotype in adult BA was significantly higher than that in adult controls (P_{cor} = 0.038). Neither the SNP nor the haplotype showing association with adult BA demonstrated a significant association with serum total immunoglobulin E (IgE) level in BA patients and controls. Our results suggest that LD block 4 confers susceptibility to adult BA with mechanisms relevant to the effector phase of allergic inflammation.

Keywords Complement · *C3* gene · Asthma · SNP · Haplotype · Association · Total IgE

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Introduction

Bronchial asthma (BA) is an inflammatory airway disease, the development of which is affected by both environmental and genetic factors (Barnes and Marsh 1998). The complement system belongs to the groups of ancient pattern-recognition systems and sensing exogenous (microorganisms) and endogenous (altered-self) danger-associated molecular patterns (Kohl and Wills-Karp 2007). The three pathways of complement activation are the classical, alternative, and lectin pathways, and all complement activation pathways converge at C3. On activation, C3 breaks into a large C3b fragment and a small C3a fragment that is called anaphylatoxin. C3b can

form C5 convertase and activate another component of the complement, C5. C5 is cleaved into C5a anaphylatoxin and C5b, and both fragments exert physiological roles. Both anaphylatoxins also show proinflammatory and immunoregulatory actions. It has been shown that complement-C3-deficient mice have reduced inflammation of asthmatic airways (Barrington et al. 2001; Kohl 2001; Muller-Eberhard 1988). Mice and guinea pigs lacking the C3a receptor (C3aR) essential for C3a action were protected against bronchoconstriction, airway hyperresponsiveness (AHR), and airway inflammation developing after allergen challenge (Bautsch et al. 2000; Drouin et al. 2002; Humbles et al. 2000). Compared with wild-type mice, C3-deficient mice also exhibit diminished AHR and lung eosinophilia when challenged with an allergen (Drouin et al. 2001). They also showed decreased numbers of interleukin (IL)-4-producing lung cells and decreased serum-antigen-specific immunoglobulin E (IgE) levels. Dendritic cells (Zhou et al. 2007) and activated T cells express C3a receptor (Werfel et al. 2000), and C3 and C3a have been shown to regulate interactions between dendritic and T cells (Kawamoto et al. 2004; Zhou et al. 2007). These results suggested that the C3 pathway is involved in allergen sensitization. In BA patients, increased levels of C3a (Humbles et al. 2000; Krug et al. 2001) and C5a (Krug et al. 2001) were observed in bronchoalveolar lavage fluid after segmental allergen provocation, suggesting involvement of the complement system in allergen-induced airway inflammation in humans. Elevation of plasma C3a level was observed in BA patients on acute exacerbation (Nakano et al. 2003). These data are consistent with the notion that the *C3* gene plays an important role in allergic sensitization and allergic inflammation. Furthermore, the *C3* gene is located at chromosome 19p, where several studies suggested linkage to BA and related phenotypes (Blumenthal et al. 2004; Lee et al. 2000; Venanzi et al. 2001). Therefore, the *C3* gene is a strong candidate gene for allergic BA.

Our previous analysis of four single nucleotide polymorphisms (SNPs) in exons of the *C3* gene suggested that variations of this gene conferred susceptibility to both childhood and adult BA (Hasegawa et al. 2004). Barnes et al. reported that a haplotype spanning introns 19–23 showed significant association with BA, log (total IgE) and log [interleukin (IL)-13]/log [interferon (IFN)-γ] (Barnes et al. 2006). The *C3* gene, however, is a large gene consisting of 41 exons and containing hundreds of SNPs; thereby, further investigation is necessary to identify functional SNPs conferring susceptibility to BA. In this study, we analyzed 26 SNPs of the *C3* gene to narrow down the regions showing association with childhood and adult BA.

Methods

Subjects

All participants were Japanese. Three hundred and forty-six childhood BA patients, 518 adult BA patients, and 550 healthy adult controls were recruited in Osaka City area, Japan. Details of these patients are described in a previous report (Nakashima et al. 2006). All participants with BA were diagnosed and selected by physicians according to the American Thoracic Society (ATS) criteria and using questionnaires based on the recommendation of the ATS, Division of Lung Disease (ATS-DLD) (Ferris 1978). In brief, patients showed repeated episodes of at least one of the following symptoms: cough, wheezing, shortness of breath, chest tightness, and sputum production. Spirometry was performed in all patients to confirm the obstructive pattern of the lung function and response to a bronchodilator. Improvement of their forced expiratory volume in 1 s (FEV_1) measurement was at least 12% after β_2 agonist inhalation. The diagnosis of atopic BA was based on one or more positive skin-scratch-test responses to a range of seven common allergens in the presence of a positive histamine control and a negative vehicle control. The seven allergens were house dust, *Felis domesticus* dander (Feld), *Canis familiaris* dander, *Dactylis glomerata*, Ambrosia, *Cryptomeria japonica*, and *Alternaria alternata*. The numbers of atopic childhood and adult BA were 304 and 371, respectively (Table 1). In this study, we only analyzed atopic BA patients. As child control subjects, 411 child volunteers with ages between 6 and 12 years (male:female = 1.0:1.04) were recruited in Chiba City, Japan. Total and eight specific IgE levels in serum were measured in this group (*Dermatophagouides pteronyssinus*, *C. familiaris*, *F. domesticus*, *A. alternata*, *C. japonica*, *D. glomerata*, egg white, golden/black bellied/Hungarian hamster). Questionnaires based on the International Study of Asthma and Allergies in Childhood (Asher et al. 1995) were used to exclude children with BA and/or atopic dermatitis. Three

Table 1 Clinical characteristics of patients and controls

	Childhood asthma	Adult asthma	Child control	Adult control
Number	304	371	333	550
Age mean (years)	9.69	45.13	9.22	44.57
Age range (years)	4–15	20–75	6–12	20–75
Gender (male:female)	1.54:1.0	1.11:1.0	1.0:1.04	2.81:1.0
Atopic asthma (%)	100	100		
Mean total IgE [log (IU/ml)]	2.66	2.54	1.90	ND

IgE immunoglobulin E, *ND* not determined

hundred and thirty-three children were used as a child control group in association studies. If atopy was defined as those who showed positive specific IgE (>0.35 IU/ml) to one or more allergens, 71% of the child controls were atopic. Details of the adult controls were described previously (Nakashima et al. 2006). Adult volunteers were interviewed by physicians, and those who were diagnosed as having BA, atopic dermatitis, and/or allergic rhinitis were excluded from the adult control group. Measurement of serum IgE levels and skin-prick tests were not performed in the adult controls. All patients and volunteers provided written informed consent to participate in the study in accordance with the rules of the process committee at the SNP Research Center (RIKEN). This study was approved by the ethics committee of Chiba University Graduate School of Medicine.

Screening and selection of SNPs

We previously investigated four SNPs in the exons of the *C3* gene (Hasegawa et al. 2004): 912G/A, 1692AG, 1936GA, and 4896 CT in our previous paper, which correspond to SNP6, SNP7, SNP13, and SNP25, respectively, in this paper (Table 2). SNP25 showed association with mite-positive childhood BA and adult BA. Because SNP25 is located in exon 41, the last exon of the *C3* gene, SNPs located around this exon were intensively investigated (SNP17–SNP26). We searched the dbSNP database to list up SNPs with the following criteria: minor allele frequency was more than 0.3, and distance of the nearest two SNPs did not exceed 2 kbp. Considering the potential importance of the gene's promoter region for gene expression, where several regulatory elements have been reported, we searched SNPs up to 5 kbp upstream of exon 1. Four SNPs (SNP1–SNP4) were identified. In addition, we included SNP14, SNP15, and SNP16 in this study to confirm the results of Barnes et al. (2006). To estimate the linkage disequilibrium (LD) status of these SNPs, we genotyped them in 96 adult controls. Haploview 3.32 program (Barrett et al. 2005) was used to show an LD map. LD block was defined with the solid spine of the LD method implemented in the Haploview program. Of the 26 SNPs investigated, 24 composed four LD blocks (Fig. 1). We selected tag SNPs from each LD block with the aid of the Tagger routine incorporated in the Haploview program.

Genotyping

Genomic DNA was prepared from whole blood samples using a standard protocol. Whole genome amplification was performed using the illustra GenomiPhi V2 amplification kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's standard protocol. Amplified deoxyribonucleic acid (DNA) was typed by allele-specific polymerase chain reaction (AS-PCR) using either the modified TaqMan AS amplification (TaqMan-ASA) method (Fujii et al. 2000) or SYBR Green detection. The primer and TaqMan probe sequences are shown in Table 3. For the TaqMan-ASA method, 2× Platinum qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA) was used as master mix, whereas 2× Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) or 2× SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) (for only SNP24) was used for AS-PCR together with SYBR Green detection. For the ASA method, the PCR mixture contained 5 μl of 2× PCR master mix, 0.4 μM of each PCR

Table 2 Locations and allele frequencies of single nucleotide polymorphisms (SNPs) of the *C3* gene

SNP	Location	Allele (1/2)[a]	Position[b]	Minor allele frequency (%)[c]	dbSNP number
SNP1	Promoter	T/C	6678365	0.330	rs171094
SNP2	Promoter	C/T	6674037	0.157	–
SNP3	Promoter	A/G	6673635	0.298	rs163913
SNP4	Promoter	A/C	6673022	0.306	rs339392
SNP5	Intron 2	A/G	6669534	0.270	rs2250656
SNP6	Exon 9	G/A	6663291	0.426	rs2230201
SNP7	Exon 14	G/A	6669848	0.468	rs2230204
SNP8	Exon 14	G/A	6663704	0.414	rs2230205
SNP9	Intron 14	G/A	6660074	0.148	rs11569429
SNP10	Intron 14	G/A	6660050	0.016	rs4807984
SNP11	Intron 17	A/G	6656246	0.403	rs11672613
SNP12	Intron 19	A/C	6648829	0.134	rs366510
SNP13	Exon 21	C/T	6648406	0.064	rs423490
SNP14	Intron 23	C/T	6647342	0.128	rs2287848
SNP15	Intron 23	C/T	6647178	0.371	rs10410674
SNP16	Intron 23	C/G	6646001	0.435	rs10402876
SNP17	Intron 33	G/A	6634846	0.436	rs344549
SNP18	Intron 33	C/G	6633953	0.441	rs344550
SNP19	Intron 33	T/–	6633534	0.446	rs11569553
SNP20	Intron 35	A/G	6631937	0.468	rs344552
SNP21	Intron 35	C/A	6631928	0.394	rs344553
SNP22	Intron 36	A/G	6630563	0.457	rs2277983
SNP23	Intron 36	G/A	6630511	0.447	rs2277984
SNP24	Intron 38	T/C	6629753	0.456	rs11569562
SNP25	Exon 41	C/T	6628989	0.447	rs4807893
SNP26	3′ Downstream	T/G	6627442	0.414	rs379527

[a] Base expressed in the direction the gene

[b] Based on National Center for Biotechnology Information (NCBI) Build 35.1 reference group label

[c] In 96 adult control subjects

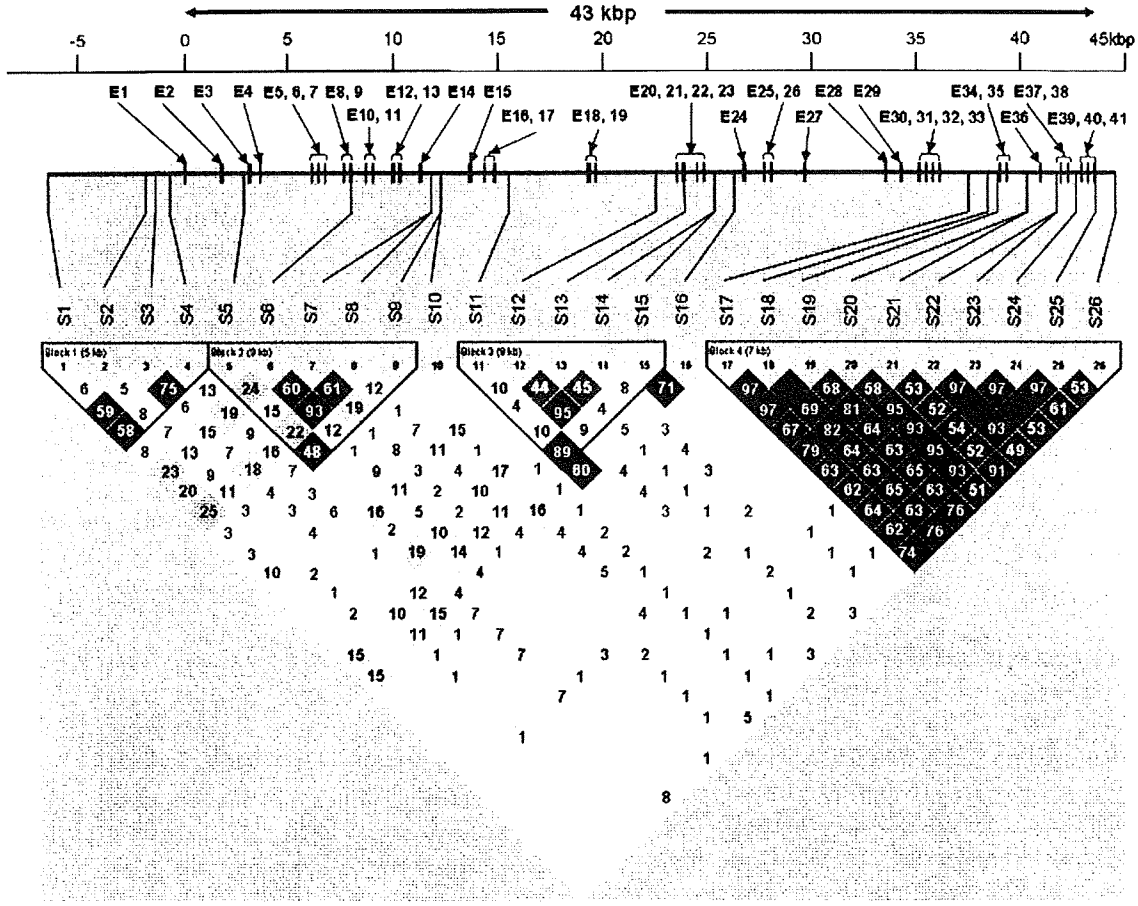

Fig. 1 Structure of the *C3* gene and location of single nucleotide polymorphisms (SNPs) and their linkage equilibrium (LD) map. Exons are indicated by *black boxes*. *Numbers* starting with *E* stand for exons and those starting with *S* for SNPs. Pair-wise LD (r^2) was estimated from 96 control subjects. LD blocks were defined by the solid spine of LD using the Haploview program. *Number* in each cell represents r^2 (×100); *black cells* $r^2 = 1$; *white cells* $r^2 = 0$. Each cell is colored in a graduated manner according to the strength of LD

primer, 0.12 μM of the TaqMan probe (when needed), and 5 ng of amplified template DNA in a final volume of 10 μl. The samples were analyzed using an ABI PRISM 7000 Sequence Detector System (Applied Biosystems, Foster City, CA, USA) and Chromo4 Real-Time System (Bio-Rad). The thermoprofile was 50°C for 2 min, 95°C for 2 min, then 45 cycles of 95°C for 15 s and 60°C for 30 s (for SNP4: 45 cycles of 95°C for 15 s, 55°C for 30 s, and 60°C for 30 s). For SNP2, PCR was initially performed using 0.2 μM of each first PCR primers and *Taq* DNA polymerase (Promega, Madison, WI, USA) according to the manufacturer's standard protocol. The thermoprofile of the first PCR was 95°C for 3 min, followed by 20 cycles at 95°C for 20 s, 60°C for 30 s, and 72°C for 7 min. In the second PCR (TaqMan-ASA method), a reaction mixture of the first PCR diluted tenfold with water was used as a template. Because an SNP was located within the sequence where the AS primer for SNP9 was designed, two primers were mixed and used in the reaction mixture for SNP9 (Table 3).

Statistical analysis

Pairwise LD in SNPs was estimated as r^2 (Hill and Robertson 1968). A 2 × 2 contingency χ^2 test of independence was performed to evaluate the significance of an association between allele frequency and disease status. Haplotype inference and a case–control association study were performed using SNPAlyze ver. 4.1 program (DYNACOM, Mobara, Japan). The effects of genotypes on $\log_{10}$-transformed total serum IgE levels were evaluated using analysis of variance (ANOVA). Statistical analysis was performed with SPSS software (ver. 15.0 J; SPSS Japan, Tokyo, Japan). In SNP association studies, corrected *P* (P_{cor}) values were calculated by multiplying the number of SNPs tested in this study (15). In haplotype association studies, P_{cor} values were calculated by multiplying the total number of haplotypes tested in the entire gene (27). Association of an SNP or haplotype with the disease was judged as significant if P_{cor} for the test of allele/haplotype frequency was <0.05.

Table 3 Primers for genotyping single nucleotide polymorphisms (SNPs) in the *C3* gene

SNP genotyped	Sequence
SNP1	
F: T allele	5′-GGCATAAACAAGGGTTAAAATGT
F: C allele	5′-GGCATAAACAAGGGTTAAAATGC
R: common	5′-GCTCACAAACGCCTATGA
TaqMan probe	5′-TGAAATAGATAAGTTGCTGCCACCCG
SNP2	
F: C allele	5′-GCCTGGCCAACATGGCGAACC
F: T allele	5′-GCCTGGCCAACATGGCGAAGT
R: common	5′-TGCCTCCTGGGTTCAAGTGATTCTC
TaqMan probe	5′-TAGTGGCGCATGCCTGTGATCCCAGCTCT
First PCR F:	5′-TTCCAAAGAGTGTGTCGCAA
First PCR R:	5′-CCTGCTTCATAGAGTTGTCGT
SNP3	
F: A allele	5′-TTTGGCAATATCTAGCAAGATTACCTA
F: G allele	5′-TTTGGCAATATCTAGCAAGATTACCTG
R: common	5′-CCTTACCACCTGCTTCATAGAGTTG
TaqMan probe	5′-TGACCCAACATATTTCCTTTCATTGCAACG
SNP4	
F: C allele	5′-CCTGTAACCTGTAAGAATGAGAC
F: A allele	5′-CCTGTAACCTGTAAGAATGAGTA
R: common	5′-CAAAGTGCTGGTGTGAACTACTG
TaqMan probe	5′-TAGTATGTGCTATGTGCTGTCC
SNP5	
R: A allele	5′-GCCTGCCCATTATTCTTGGTCCT
R: G allele	5′-GCCTGCCCATTATTCTTGGTCAC
F: common	5′-CCTTGTGAGCTCTTCTTTTTGAGTTC
SNP6	
R: G allele	5′-CACCCCGTCCAGCAGTACCTAC
R: A allele	5′-CACCCCGTCCAGCAGTACCTAT
F: common	5′-AACAGAGGATTTCCCTGCCTGAA
TaqMan probe	5′-CCCTCAAGCGCATTCCG
SNP7	
F: A allele	5′-AACAGAGGATTTCCCTGCCTGTA
F: G allele	5′-AACAGAGGATTTCCCTGCCTGGG
R: common	5′-CACCCCGTCCAGCAGTACCTTC
TaqMan probe	5′-CCCTCAAGCGCATTCCG
SNP8	
F: G allele	5′-TGCTGAATAAGAAGAACAAACTGAGG
F: A allele	5′-TGCTGAATAAGAAGAACAAACTGAGA
R: common	5′-TGCCTCCGCCTCTTCTCA
SNP9	
F: G allele	5′-TAGGGACGAAGATGGAGATGTG
G allele	5′-TAGGGACGAAGATGGAGATGAG
F: A allele	5′-TAGGGACGAAGATGGAGATGTA
A allele	5′-TAGGGACGAAGATGGAGATGAA
R: common	5′-CTTATCTCCATTTCCCCTCTGATTC
SNP10	
R: G allele	5′-TGATTCCATCTGCATTCCCAAC
R: A allele	5′-TGATTCCATCTGCATTCCCAAT
F: common	5′-TTTCCGGAGTAGGGACGAAGA

Table 3 continued

SNP genotyped	Sequence
SNP11	
F: A allele	5′-AGGGTCACTGGGAAAATTAGACATA
F: G allele	5′-AGGGTCACTGGGAAAATTAGACAGG
R: common	5′-ATGGGCCAAAGGAATTACACAAT
SNP12	
F: A allele	5′-ACTCCCCGACCTTGACACTAA
F: C allele	5′-ACTCCCCGACCTTGACACTAC
R: common	5′-CCTGCATTACTGTGACCTCGAA
TaqMan probe	5′-CCCGAGCAGGGATCTGTGTGGCA
SNP13	
F: C allele	5′-GGAAGTGGAAGTCAAGGCTGGC
F: T allele	5′-GGAAGTGGAAGTCAAGGCTGGT
R: common	5′-GGGTGCCCCAAGCACTCA
TaqMan probe	5′-CCATCATTTCATCAGTGACGGTGTCAGGAA
SNP14	
R: T allele	5′-GAATGAGATGGAATTTGGCTCGA
R: C allele	5′-GAATGAGATGGAATTTGGCTCTG
F: common	5′-CAAGTCCCGGACACCGAGTCT
SNP15	
R: C allele	5′-CAGCGAGCTGAGGTCGGG
R: T allele	5′-CAGCGAGCTGAGGTCGTA
F: common	5′-CATCTGTGATCTGTTTTCCCTCTTTTAC
SNP16	
R: C allele	5′-GAGTGTCTCACTTAATAGTCAACGATG
R: G allele	5′-GAGTGTCTCACTTAATAGTCAACGATC
F: common	5′-TGGTCAGGCTGGTCTTGAACTC
SNP17	
F: G allele	5′-CTGCCAAAGTTTTGGGATCACTG
F: A allele	5′-CTGCCAAAGTTTTGGGATCACTA
R: common	5′-CCACACCCGGCCATTTCC
SNP18	
R: C allele	5′-AATGCCAGAAGTGAACTTCAAAGTG
R: G allele	5′-AATGCCAGAAGTGAACTTCAAAGTC
F: common	5′-CAGCAGGGTCAACATCACCATA
SNP19	
F: T allele	5′-GGCTGCCTGTATTCTTGCCTAT
F: delT allele	5′-GGCTGCCTGTATTCTTGCCTCG
R: common	5′-TGGATTCAAATTCCAGCTCTAAATAAC
SNP20	
F: A allele	5′-ATTCCAAGCATGAGCCACGA
F: G allele	5′-ATTCCAAGCATGAGCCACGG
R: common	5′-GGAGAGGAGAAAGCCCAAATCA
SNP21	
R: A allele	5′-GATGGAGAGAAAATAACAGAAGAGTT
R: C allele	5′-GATGGAGAGAAAATAACAGAAGAGCG
F: common	5′-ATGTTGCTCAAGTTGGTCTCAAACT
SNP22	
R: A allele	5′-GGCCTCCCTCCAAAGACCCT
R: G allele	5′-GGCCTCCCTCCAAAGACCTC
F: common	5′-CGTGTCCCAGGAATCTATGAATTT

Table 3 continued

SNP genotyped	Sequence
SNP23	
R: G allele	5′-ACCGGGTACAGCTTTCCTCTAC
R: A allele	5′-ACCGGGTACAGCTTTCCTCTTT
F: common	5′-GGCTTCTGTGAGTTGAGAGTCTAAGAGA
SNP24	
F: T allele	5′-CATGGCCATGAGGCTACAGTATAT
F: C allele	5′-CATGGCCATGAGGCTACAGTATAC
R: common	5′-CCCATGTCACCATCCACACA
SNP25	
F: T allele	5′-ACACTTGGGTGGAGCACTGGCAT
F: C allele	5′-ACACTTGGGTGGAGCACTGGCTC
R: common	5′-GGTCCTGGCATTGTTTCTGGTTCTC
TaqMan probe	5′-AGGAGGACGAATGCCAAGACG
SNP26	
R: T allele	5′-GGTGAGAATGTGGGCAAGAAGA
R: G allele	5′-GGTGAGAATGTGGGCAAGAAGC
F: common	5′-ACCTACATCCTCTCCGGTGAGTGT

PCR polymerase chain reaction, *F* forward primer, *R* reverse primer. All TaqMan probes were labeled with 6-carboxyfluorescein (FAM, reporter dye) at the 5′ end and 6-carboxy-tetramethyl-rhodamine (TAMRA, quenching dye) at 3′ end

Results

Polymorphisms in the *C3* gene

We selected and characterized 26 SNPs from the *C3* gene to investigate mainly genetic variations of the promoter region, SNPs forming a haplotype that showed significant association with BA in African Caribbean families, and the 3′ end region where a SNP showed significant association with BA in our previous study (Table 2). The location of the SNPs and LD map is shown in Fig. 1. If an LD block was defined by the solid spine of LD, out of the 26 SNPs investigated, 24 composed four LD blocks. SNP10 showed r^2 values <0.033 to any other SNPs investigated in the current study and excluded from any LD block under this definition. Although SNP16 showed moderate LD to SNP15 ($r^2 = 0.71$) and SNP11 ($r^2 = 0.60$), it did not belong to LD block 3, where SNP15 and SNP11 are located. This is because of the characteristic of the "solid spin of LD" definition. When SNP12 and SNP14, both of which showed very low r^2 (<0.01) to SNP16, were omitted from LD block 3, SNP16 was found to be included in LD block 3. As tag SNPs, four SNPs each were selected in LD blocks from 1 to 3 and three SNPs in LD block 4 (Fig. 1).

Association study of childhood and adult BA

We genotyped 15 tag SNPs in 304 childhood BA, 371 adult BA, 333 child controls, and 550 adult controls (Table 1). All loci were in Hardy–Weinberg equilibrium in the control groups. The genotype and allele frequencies of each SNP in the patient and control groups are shown in Table 4. Results of association tests for allele frequency between the patient and control groups are also shown. Allelic frequency of SNP24 was significantly different between adult BA and adult controls ($P = 0.002$). The P value for the difference remained significant ($P_{cor} = 0.030$) after correction for the number of SNPs tested (15). In a recessive model, the odds ratio (OR) and its 95% confidence interval (95% CI) range for this SNP were 1.55 and 1.15–2.09, respectively. In a dominant model, the OR and 95% CI range were 1.37 and 1.01–1.88, respectively. The difference in the allele frequency of SNP24 between all BA and all control showed a similar tendency. However, the P value was not significant ($P = 0.004$, $P_{cor} = 0.060$). None of the other SNPs showed a significant association with either childhood or adult BA.

The frequencies of haplotypes consisting of tag SNPs in four LD blocks are shown in Table 5. A significant difference was observed in LD block 4 (Table 5). In LD block 4 containing exons 24–41, the frequency of the major haplotype CCC in adult BA (54.1%) was significantly higher than that in controls (46.4%) ($P = 0.0014$, $P_{cor} = 0.038$). The frequencies of C alleles of SNP18, SNP19, and SNP24 were higher in adult BA than in adult controls. These increased frequencies were straightforwardly associated with the increased frequency of the CCC haplotype. The frequency of this haplotype in childhood BA (51.0%) was not significantly higher than that in adult controls (48.6%) ($P_{cor} > 1.0$). The OR of the CCC haplotype of LD block 4 was about 1.4 for both childhood and adult BA (Table 6). In LD block 2 containing at least exons 3–14, the frequency of the GGAG haplotype in childhood BA (5.6%) was higher than that in child controls (2.5%) ($P = 0.0044$). This difference, however, did not reach a gene-wide significance ($P_{cor} = 0.12$). In LD blocks 1 and 3, there was no haplotype showing any difference in frequency between patients and controls. LD block 1 contains the promoter region of the *C3* gene; therefore, it is not likely that genetic variations of the promoter region have significant effect on susceptibility to BA in the Japanese population.

Barnes et al. reported that a 3-SNP haplotype consisting of the SNPs identical to SNP14, SNP15, and SNP16 showed significant association with BA, log (total IgE) and log (IL-13)/log (IFN-γ) in the Afro-Caribbean families (Barnes et al. 2006). To assess the reproducibility of their results, we investigated whether this 3-SNP haplotype shows association with either childhood BA, adult BA, or log (total IgE) in our samples. We found that this haplotype showed no significant association with any of these phenotypes.

Table 4 Single nucleotide polymorphism (SNP) association study of the *C3* gene

	Childhood asthma					Adult asthma					Child control					Adult control					Childhood asthma versus child control	Adult asthma versus adult control	All asthma versus all control
	11[a]	12[b]	22[c]	1[d]	2[d]	11[a]	12[b]	22[c]	1[d]	2[d]	11[a]	12[b]	22[c]	1[d]	2[d]	11[a]	12[b]	22[c]	1[d]	2[d]	P_{cor}[e]	P_{cor}[e]	P_{cor}[e]
SNP1	0.382	0.473	0.144	0.619	0.381	0.438	0.432	0.130	0.654	0.346	0.351	0.508	0.141	0.605	0.395	0.395	0.457	0.149	0.623	0.377	>1	>1	>1
SNP2	0.755	0.222	0.024	0.865	0.135	0.785	0.171	0.044	0.871	0.130	0.754	0.192	0.055	0.849	0.151	0.769	0.205	0.027	0.871	0.129	>1	>1	>1
SNP3	0.440	0.443	0.117	0.662	0.339	0.431	0.456	0.113	0.659	0.341	0.375	0.502	0.123	0.626	0.374	0.424	0.467	0.109	0.658	0.343	>1	>1	>1
SNP4	0.433	0.406	0.161	0.636	0.364	0.437	0.439	0.124	0.657	0.344	0.402	0.475	0.123	0.640	0.361	0.426	0.430	0.143	0.642	0.358	>1	>1	>1
SNP5	0.542	0.405	0.054	0.744	0.256	0.616	0.323	0.061	0.778	0.223	0.562	0.369	0.069	0.747	0.254	0.628	0.310	0.062	0.783	0.217	>1	>1	>1
SNP6	0.411	0.461	0.128	0.642	0.359	0.371	0.454	0.175	0.598	0.402	0.381	0.469	0.150	0.616	0.385	0.327	0.485	0.188	0.570	0.431	>1	>1	>1
SNP7	0.201	0.497	0.302	0.450	0.551	0.256	0.497	0.247	0.505	0.496	0.255	0.482	0.264	0.496	0.504	0.285	0.485	0.230	0.528	0.473	>1	>1	>1
SNP9	0.737	0.232	0.030	0.854	0.146	0.750	0.219	0.031	0.860	0.141	0.709	0.273	0.018	0.846	0.155	0.753	0.219	0.028	0.863	0.138	>1	>1	>1
SNP11	0.340	0.505	0.155	0.593	0.408	0.331	0.489	0.180	0.576	0.425	0.324	0.502	0.174	0.575	0.425	0.312	0.478	0.210	0.551	0.449	>1	>1	>1
SNP13	0.862	0.131	0.007	0.928	0.073	0.889	0.106	0.006	0.941	0.059	0.880	0.120	0.000	0.940	0.060	0.890	0.110	0.000	0.945	0.055	>1	>1	>1
SNP14	0.769	0.228	0.003	0.883	0.117	0.779	0.202	0.019	0.880	0.120	0.778	0.213	0.009	0.885	0.116	0.786	0.206	0.008	0.889	0.111	>1	>1	>1
SNP15	0.379	0.480	0.141	0.619	0.381	0.348	0.514	0.138	0.605	0.395	0.363	0.469	0.168	0.598	0.403	0.316	0.503	0.181	0.568	0.433	>1	>1	>1
SNP18	0.305	0.557	0.138	0.584	0.417	0.403	0.442	0.156	0.623	0.377	0.348	0.454	0.198	0.575	0.425	0.328	0.506	0.167	0.580	0.420	>1	>1	>1
SNP21	0.367	0.522	0.111	0.628	0.372	0.433	0.444	0.122	0.656	0.344	0.369	0.471	0.159	0.605	0.395	0.358	0.498	0.144	0.607	0.393	>1	0.540	0.555
SNP24	0.248	0.537	0.215	0.517	0.484	0.225	0.456	0.319	0.453	0.547	0.282	0.450	0.267	0.508	0.492	0.285	0.482	0.233	0.526	0.474	>1	0.030	0.060

[a] Frequency of homozygote for major allele

[b] Frequency of heterozygote

[c] Frequency of homozygote for minor allele

[d] Allele frequency

[e] Corrected *P* value (raw *P* values were multiplied by number of SNPs, 15) for allele frequency difference

Table 5 Haplotype association study of the *C3* gene

Haplotype	Frequency				P_{cor}[a]		
	Childhood asthma	Adult asthma	Child control	Adult control	Childhood asthma vs. control	Adult asthma vs. control	All-asthma vs. control
Block 1[b]							
TCAA	0.441	0.483	0.436	0.467	>1	>1	>1
CCGC	0.295	0.263	0.279	0.280	>1	>1	>1
TTAA	0.129	0.108	0.121	0.103	>1	>1	>1
CCAA	0.051	0.043	0.041	0.042	>1	>1	>1
TCGC	0.032	0.051	0.040	0.021	>1	0.222	>1
CCGA	0.009	0.014	0.032	0.020	0.192	>1	0.297
CCAC	0.019	0.010	0.019	0.016	>1	>1	>1
TCAC	0.018	0.009	0.004	0.017	0.324	>1	>1
Others	0.006	0.022	0.029	0.027			
Block 2[c]							
AAGG	0.322	0.361	0.333	0.403	>1	>1	>1
AGAG	0.337	0.301	0.310	0.291	>1	>1	>1
GGAA	0.124	0.110	0.141	0.115	>1	>1	>1
AGGG	0.058	0.067	0.072	0.063	>1	>1	>1
GGGG	0.049	0.055	0.053	0.051	>1	>1	>1
GGAG	0.056	0.042	0.025	0.037	0.121	>1	0.729
AAAG	0.016	0.018	0.015	0.015	>1	>1	>1
AGAA	0.014	0.024	0.013	0.010	>1	0.567	>1
GAGG	0.019	0.015	0.036	0.009	>1	>1	>1
Others	0.006	0.006	0.002	0.005			
Block 3[d]							
ACCC	0.460	0.446	0.445	0.424	>1	>1	>1
GCCT	0.365	0.382	0.384	0.418	>1	>1	>1
ATTC	0.066	0.058	0.055	0.054	>1	>1	>1
ACTC	0.049	0.059	0.057	0.055	>1	>1	>1
GCCC	0.041	0.041	0.038	0.034	>1	>1	>1
ACCT	0.013	0.012	0.014	0.013	>1	>1	>1
Others	0.006	0.002	0.006	0.003			
Block 4[e]							
CCC	0.510	0.541	0.486	0.464	>1	0.038	0.079
GAT	0.365	0.340	0.387	0.375	>1	>1	>1
CCT	0.066	0.078	0.081	0.098	>1	>1	>19
GCT	0.053	0.033	0.038	0.044	>1	>1	>1
Others	0.007	0.011	0.008	0.019			

[a] Corrected *P* value (raw *P* values were multiplied by the number of haplotypes tested, 27) for haplotype frequency difference

[b] This haplotype is consisted of SNPs 1, 2, 3, and 4

[c] This haplotype is consisted of SNPs 5, 6, 7, and 9

[d] This haplotype is consisted of SNPs 11, 13, 14, and 15

[e] This haplotype is consisted of SNPs 18, 21, and 24

Table 6 Odds ratio (OR) and its 95% confidence interval (CI) of the CCC haplotype for bronchial asthma (BA)

	Diplotype frequency (%)				
	Case		Control		
	CCC/CCC and CCC/other	Other/other	CCC/CCC and CCC/other	Other/other	Odds ratio (95% CI)
Childhood BA	77.8	22.2	71.2	28.8	1.42 (0.99–2.04)
Adult BA	76.9	23.1	71.0	29.0	1.36 (1.00–1.86)
All BA	77.3	22.7	71.1	28.9	1.39 (1.10–1.75)

Diplotype of each individual was inferred with SNPAlyze ver. 4.1. OR and its (95% CI) were determined with SPSS ver. 15.0

Effect of genetic variations of the *C3* gene on serum total IgE level

To examine the effect of genetic variations of the *C3* gene on serum IgE level, 15 tag SNPs and the CCC haplotype were analyzed by analysis of variance (ANOVA) for log-transformed serum total IgE values (Table 7). *P* values <0.05 were observed in SNP14 in adult BA patients (P = 0.042) and in SNP13 (P = 0.018) and SNP18 (0.041) in child controls. If we want to maintain type 1 error at 0.05 in each patient/control group, a *P* value <0.0031 (0.05/16) should be considered as significant. Thus, we deduced that these SNPs did not significantly affect serum total IgE level. The genotype of SNP24 and the CCC haplotype, both of which showed an association with adult BA, were not significantly associated with the IgE level.

Table 7 Effect of single nucleotide polymorphisms (SNPs) and haplotypes of the *C3* gene on $\log_{10}$-transformed total immunoglobulin E (IgE)

Locus	*P* value in ANOVA test[a]		
	Childhood asthma	Adult asthma	Child control
Block 1			
SNP1	0.347	0.242	0.250
SNP2	0.657	0.960	0.320
SNP3	0.276	0.051	0.720
SNP4	0.329	0.058	0.182
Block 2			
SNP5	0.537	0.794	0.119
SNP6	0.860	0.533	0.805
SNP7	0.952	0.556	0.497
SNP9	0.368	0.795	0.270
Block 3			
SNP11	0.463	0.678	0.937
SNP13	0.917	0.100	0.018
SNP14	0.485	0.042	0.261
SNP15	0.486	0.816	0.688
Block 4			
SNP18	0.197	0.890	0.041
SNP21	0.065	0.280	0.097
SNP24	0.356	0.661	0.526
CCC haplotype	0.175	0.586	0.505

[a] Significance of difference in the mean of $\log_{10}$ (total IgE) among individuals with different genotypes (major allele homozygote, heterozygote, minor allele homozygote) was tested with analysis of variance (ANOVA). Three groups (childhood asthma, adult asthma, and child control) were evaluated separately. Serum IgE level was not determined in the adult control group

Discussion

In this study, we investigated the association of SNPs in the *C3* gene with childhood and adult BA. We observed a significant association of SNP24 and the CCC haplotype in LD block 4 with adult BA. As the CCC haplotype was discriminated from other major haplotypes with a frequency of >1% by the SNP24 allele (C/T), this haplotype association may just be a reflection of the association of SNP24. There was no significant difference in genotype frequency of SNP24 between the adult control group and the child control group, suggesting that the significant association observed in the adult patients–control comparison was not due to skewed genotype frequency of adult controls but to changes in adult BA. The frequency of the T/T genotype in childhood BA was decreased (from 0.282 to 0.248), as in adult BA. However, the change in allele frequency in childhood BA compared with that in child controls was small (T allele: 0.517 vs. 0.508). This SNP (or that with strong LD to this SNP) may not be a risk-modifying variation for childhood BA. Although it was not significant at the gene-wide level, we observed a tendency of the frequency of the GGAG haplotype of LD block 2 to show a difference between childhood BA and child controls (P = 0.0045, P_{cor} = 0.121). To determine and definitively conclude whether this LD block confers risk for childhood BA, an association study with more childhood samples and functional analyses of this region will be necessary.

LD block 4 is about 20 kb long and contains exon 41 coding the C-terminal end of the C3 protein and 3′ untranslated region as well as upstream 17 exons and introns. Because we could not find SNPs that change the amino acid sequences in exons in this LD block, functional variation(s) in this region should be those affecting either expression of the gene, ribonucleic acid (RNA) splicing, or RNA stability. To the best of our knowledge, there is as yet no study investigating elements affecting gene expression in this region of the *C3* gene. Currently, we do not have sufficient data to discuss the underlying mechanisms of the association between the LD block and adult BA. Further genetic and functional analyses of the LD blocks are necessary to pinpoint genetic variation(s) responsible for differences in susceptibility to BA.

It is possible that some of the control subjects involved in this study will develop asthma. Judging from the prevalence of the asthma in Japan, the chance of developing the disease is expected to be several percent. If we exclude atopic subjects from the control group, the chance may be reduced but not completely eliminated. This issue cannot be thoroughly controlled in a case–control study. The fact that our control group contained presymptomatic BA patients reduces the statistical power to detect an association between genotypes and the disease. We must be aware of this issue

when obtaining negative results. However, the main result (i.e., the significant association of the haplotype with the disease) cannot be changed by this issue. If we could eliminate presymptomatic BA patients, estimated ORs would be higher than the values we presented in this study.

We also investigated whether genetic variations of the *C3* gene affect total serum IgE level using childhood and adult BA patients and child controls. The IgE level was not significantly affected by any SNPs or haplotypes, including those showing a significant association with adult BA. This was true with mite-specific IgE level (data not shown). These results suggest that genetic variations of LD block 4 showing susceptibility to BA have a slight effect on sensitization to allergens but are more relevant to the effector phase of allergic inflammation. The roles of anaphylatoxins in the pathogenesis of BA can be divided into two phases: sensitization to allergens and effector phase of allergic BA (Kohl and Wills-Karp 2007). Several roles of C5a signaling at the interface between dendritic cells and T cells are evident, but those of C3a remain unclear. In a C3aR knockout experiment, different Th2 cytokine production responses were reported in various strains of mice (C57BL/6 and BALB/c) (Drouin et al. 2002; Humbles et al. 2000), suggesting that the effect of C3a is influenced by genetic background. As observed in mice, a difference in genetic background may explain the fact that association of the haplotype consisting of SNP14, SNP15, and SNP16 with total IgE level observed in Afro-Caribbean families was not confirmed in the Japanese population. Discrepancy between our results and those of Barnes et al. may also be due to a difference in the environmental factors of the two study populations. There are a number of studies showing that the effects of genetic variation (−159C/T) of the CD14 gene on allergic sensitization and BA risk differ greatly due to environmental factors such as mite or lipopolysacharide concentration in dust (Ober et al. 2000; Simpson et al. 2006; Vercelli 2003; Zambelli-Weiner et al. 2005). It may be possible that *C3* gene variations also show this type of gene–environmental interaction and cause discrepant results in studies with different populations.

In summary, our results suggest that the LD block containing exons 24–41 of the *C3* gene confer susceptibility to adult BA in the Japanese population. Because this region showed a slight effect on serum IgE level in both BA patients and normal individuals, this region may be involved in the effector phase of allergic inflammation. The effect of variations of the *C3* gene on allergic sensitization and BA susceptibility may differ according to genetic background and environmental factors.

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Migration of Tumor Antigen-Pulsed Dendritic Cells After Mucosal Administration in the Human Upper Respiratory Tract

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Abstract Tumor-specific peptide-pulsed dendritic cells (DC) were administered via different routes to a group of patients with head and neck cancers. The migration and homing patterns of such antigen-stimulated cells was carefully studied employing single photon emission computed tomography (SPECT). The DC administered directly into the nasal submucosa quickly migrated very rapidly to the regional neck lymph nodes in the neck. However, after inoculation of the cells into the palatine tonsils, the DCs remained close to the site of administration and did not migrate to the regional lymph nodes or to other mucosal regions. After nasal submucosal administration of the DC, tumor-antigen-specific cytotoxic T cells were detected in the ipsilaterals but not in the contra lateral lymph nodes. These results suggest that after antigen processing, the regional lymph nodes serve as inductive sites for development of mucosal immune responses and for induction of memory cells during the local immunological responses in the nasopharyngeal-associated lymphoid tissue in man.

Keywords Dendritic cell · migration · mucosal vaccines · human studies · cancer vaccines

Abbreviations

DC	dendritic cell(s)
NALT	nasopharyngeal-associated lymphoid tissue
SPECT	single photon emission computed tomography
ROI	region of interest
HLA	human leukocyte antigen
CTL	cytotoxic T lymphocyte

Introduction

A distinct nasal-associated lymphoid tissue (NALT) located in the anterior nasal cavity is an important inductive site of mucosal immune responses in the rodents and functions in a similar manner to the Peyer's patches in the gut and other organized lymphoid tissues [1–4]. The nasopharyngeal tonsils and the salivary glands and other tissues, collectively referred to as Waldeyer's Ring, appear to represent the equivalent of NALT in man. It includes isolated lymphoid follicles having an overlying lymphoepithelium with M cells [5–8]. Recently, it has been proposed that the upper respiratory tract could be an attractive vaccine administration route because of its ability to mount effective mucosal as well as systemic immune responses [9–13]. However, the role of such immunization in cancer immunotherapy remains to be elucidated.

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