

Seasonal changes of humoral and cellular immune responses to Japanese cedar (*Cryptomeria japonica*) pollen allergens in Japanese monkeys (*Macaca fuscata*) with pollinosis

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Abstract: The natural occurrence of Japanese cedar [*Cryptomeria japonica* (CJ)] pollinosis has been reported in Japanese monkeys (*Macaca fuscata*). The present study was designed to investigate seasonal changes in immunological reactions to CJ pollen allergens in monkeys with CJ pollinosis. Blood samples were collected from six monkeys with CJ pollinosis before and after CJ pollen season. Seasonal changes in specific IgE and IgG to major allergens (Cry j 1 and Cry j 2) were observed before and after CJ pollen season. The humoral responses decreased significantly before CJ pollen and increased after CJ pollen season. Similar seasonal changes in peripheral blood mononuclear cells proliferative responses to CJ allergens were observed before and after CJ pollen season. These humoral and cellular immune responses might serve as a biomarker for assessing new immunotherapies for monkeys with pollinosis.

**Masahiro Sakaguchi¹,
Tomoko Yamada³,
Kazuki Hirahara⁴,
Akio Shiraishi⁴, Saburo Saito⁵,
Hiroshi Miyazawa⁶,
Yoshifumi Taniguchi⁷,
Sakae Inouye², Hideo Nigi⁸**

¹Department of Immunology and ²Infectious Disease Surveillance Center, National Institute of Infectious Diseases, ³Division of Wild Animal Medicine, Nippon Veterinary and Animal Science University, ⁴Biological Research Laboratories, Sankyo Co., Ltd., ⁵Institute of DNA Medicine, Jikei University School of Medicine, ⁶Department of Medical Technology, Kyorin University, Tokyo, ⁷Hayashibara Biochemical Laboratories, Okayama, ⁸Faculty of Human Sciences, Osaka University, Suita, Japan

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Dr. Masahiro Sakaguchi, Department of Immunology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162, Japan.
E-mail: msakaguc@nih.go.jp

Introduction

Japanese cedar [*Cryptomeria japonica* (CJ)] pollinosis is one of the most common allergic diseases in Japan [11, 19], with an increasing number of patients [25]. A recent study reported that the prevalence of CJ pollinosis is 11% of the Japanese population [24].

Two major allergens have been isolated from CJ pollen: Cry j 1 [29] and Cry j 2 [20]. It was reported that five amino acid residues of the N-terminus of Cry j 1 were identical to those of the mountain cedar, which grows in North America and belongs to the same order (Pinales) as Japanese cedar [27].

The c-DNA of Cry j 1 has been cloned and the Cry j 1 sequence shows some homology to Amb a 1 and Amb a 2 family of genes for ragweed pollen allergens [5, 26]. Cry j 2 c-DNA was also obtained [13, 15], and further investigation has revealed that the Cry j 2 sequence shows a significant homology with that of tomato polygalacturonase and also that Cry j 2 has polygalacturonase enzyme activity [17].

The natural occurrence of Japanese cedar pollinosis has also been reported in Japanese monkeys (*Macaca fuscata*) [21, 30], in which the symptoms resemble those of human patients. In addition, the

monkeys with pollinosis had specific IgE to Cry j 1 and Cry j 2 [6, 21]. Furthermore, leukocytes from monkeys with pollinosis demonstrated pollen allergen-specific histamine release, as well as a significant proliferation of peripheral blood mononuclear cells (PBMC), in response to the pollen allergens [8]. Japanese monkeys have been well established as a suitable animal model for studying CJ pollinosis.

Seasonal changes in immunological responses to some pollen allergens have been reported in human patients [1, 2, 4, 31]. In addition, some studies have discussed seasonal changes in the immune responses to CJ allergens in human patients [7, 10, 16]. However, seasonal changes in the immunological responses to allergens in nonhuman primates have not yet been reported.

To investigate seasonal changes in the immunological response to CJ pollen allergens in monkeys with CJ pollinosis, we examined specific IgE, IgG, and PBMC proliferative responses to CJ pollen allergens.

Materials and methods

Antigen

Pollen was extracted in 0.125 M ammonium bicarbonate (pH 8.0) at 4°C for 48 hours. After removal of the precipitate by centrifugation, the supernatant was lyophilized. The lyophilized one was prepared as pollen crude extract [29]. Cry j 1 and Cry j 2 were purified from Japanese cedar (CJ) pollen as described previously [20, 29].

Monkeys

Blood samples were collected from six female Japanese monkeys (nos. 1–6) ranging from 4 to 24 years old with CJ pollinosis in a free-ranging troop in Awaji Island, Hyogo Prefecture, Japan. As a control, blood samples from two monkeys (nos. 7 and 8) in this troop that had no CJ pollinosis and no anti-CJ IgE were prepared. We came daily to the feeding place and examined the presence of pollinosis symptoms such as tear production, eye redness, sneezing, and rhinorrhea in the monkeys [22, 23]. All the monkeys were named and identified. This monkey troop is the only one on this island [6, 22, 23]. CJ pollen season in this area is from February to April. We collected sera from these monkeys before (December) and after (May) the pollen season. A mixture of ketamine HCl (Ketalar; Sankyo, Tokyo, Japan) at about 5 mg/kg body weight and xylazine HCl (Celactar; Bayer, Tokyo, Japan) at about 1 mg/kg body weight was administered intramuscularly by blowgun to each

monkey based on a rough estimation of body weight. After each monkey was examined, 4 mg/kg of torazoline HCl (Sigma Chemicals, St. Louis, MO) was given intravenously or intramuscularly as an antagonist to xylazine HCl. Awakened monkeys were released. All serum specimens were stored at –70°C until use.

Measurement of specific IgE to crude pollen allergens

The CAP method (Amersham Pharmacia Biotech, Uppsala, Sweden) was used according to the manufacturer's instructions to measure specific IgE to CJ pollen allergens [6, 12].

Measurement of specific IgE to two major allergens

Specific IgE to Cry j 1 and Cry j 2 were measured by a fluorometric ELISA as described previously [6]. Briefly, Cry j 1 or Cry j 2 (1 µg/ml) was added to microplate wells for immobilization. After incubation of the monkey serum (diluted 1:8) with the immobilized antigens, anti-human IgE antibody conjugated with β-D-galactosidase (Amersham Pharmacia Biotech) was diluted to 1:25 and was added to each well. As the enzyme reaction substrate, 0.2 mM 4-methylumbelliferyl-β-D-galactoside (Sigma Chemicals) was added and the plates were incubated at 37°C for 2 hours. Then, 0.1 M glycine-NaOH (pH 10.3) was added to stop the reaction. Fluorescence units (FU) were measured on a fluorometric microplate reader (Fluoroskan; Flow Laboratories, McLean, VA). The specific IgE level was expressed as arbitrary units (U)/ml calculated from a titration curve of an antibody-positive reference serum.

Measurement of specific IgG to two major allergens

Specific IgG to Cry j 1 and Cry j 2 were assayed by a reverse-sandwich ELISA as described previously [14]. Briefly, microplates were coated with Cry j 1 or Cry j 2 (1 µg/ml) in 0.05 M carbonate–bicarbonate buffer pH 9.6, containing 0.5 M NaCl, and 20 µg/ml of bovine serum albumin was added to coat the microplate wells. After incubation of diluted monkey serum with the immobilized antigens, the wells were reacted with biotinylated Cry j 1 or Cry j 2 (0.5 µg/ml). Next, β-D-galactosidase-conjugated streptavidin (Zymed Laboratories, San Francisco, CA) was added to each well. The following procedures were the same as those used in the above-mentioned fluorometric ELISA. The specific IgG level was expressed as U/ml calculated from a titration curve of an antibody-positive reference serum.

The IgG specificity of the reverse-sandwich ELISA was assessed by absorption experiment using protein G. When pooled serum of monkeys with CJ pollinosis was incubated with protein G-Sepharose 4B (Zymed Laboratories), anti-Cry j 1 and Cry j 2 IgG activity in the serum was completely reduced, but anti-Cry j 1 and Cry j 2 IgE activity was not changed (data not shown).

PBMC proliferative response to major allergens

PBMC proliferative response to major allergens was carried out as previously described [8]. Briefly, fresh heparinized venous blood was taken from monkeys with CJ pollinosis. This blood was incubated with 1% dextran (Amersham Pharmacia Biotech) and isolated at room temperature for 2 hours. The supernatants were then centrifuged and the pellet was resuspended. The PBMCs from these cells were isolated by Ficoll (Amersham Pharmacia Biotech) and the cell viability was more than 90%. PBMCs were cultured at 5×10^5 cell/well in 96-well flat bottom plates with and without Cry j 1 or Cry j 2 (1 $\mu\text{g/ml}$) for 3 days at 37°C. RPMI medium 1640 (Gibco BRL, Grand Island, NY) and 10% human serum type AB (Biological Specialty Co., Lansdale, PA) as culture medium were used. Each well was then pulsed with 0.5 μCi of

^3H -thymidine (Amersham Pharmacia Biotech), and the cells were harvested 16 hours later. The radioactivity was measured; each sample was prepared in either duplicate or triplicate. The results, indicated as a stimulation index (SI), were calculated as follows: mean counts per minute (CPM) in the presence of antigen divided by mean CPM in the absence of antigen.

Statistical analysis

Specific IgE, IgG, and PBMC proliferative response to CJ allergens before and after the CJ pollen season were compared using a paired *t*-test with $P < 0.05$ considered as significant.

Results

Seasonal changes in specific IgE levels to crude CJ allergens

Figure 1 shows seasonal changes in specific IgE levels to crude CJ allergens in six monkeys with CJ pollinosis before and after CJ pollen season. After CJ pollen season in May 1997, all monkeys had specific IgE to crude CJ. As a control, two monkeys without CJ pollinosis had no specific IgE to crude CJ (data not shown). Before CJ pollen season (December 1997), the levels of specific IgE to

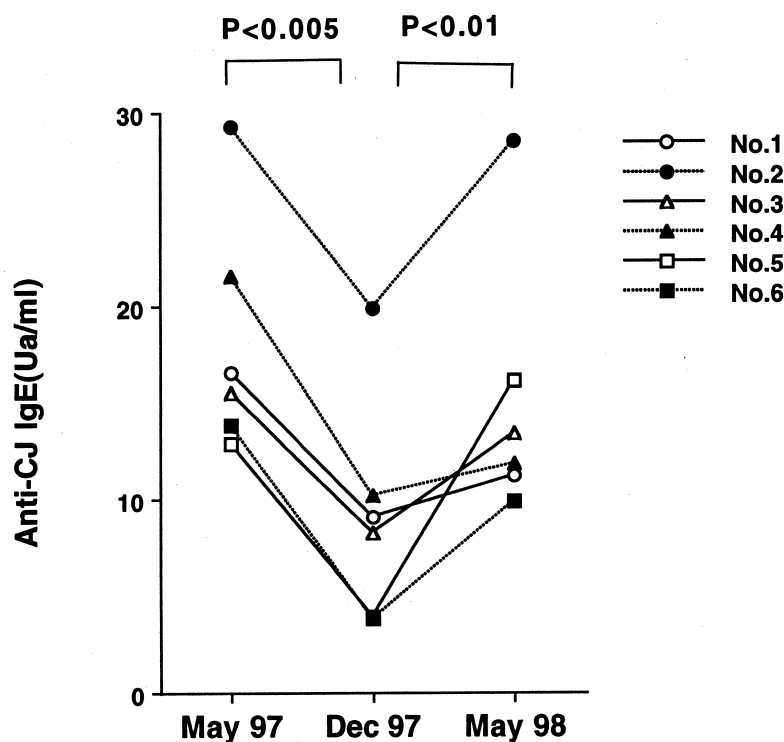


Fig. 1. Seasonal changes in specific IgE to crude CJ allergens in the monkeys with CJ pollinosis.

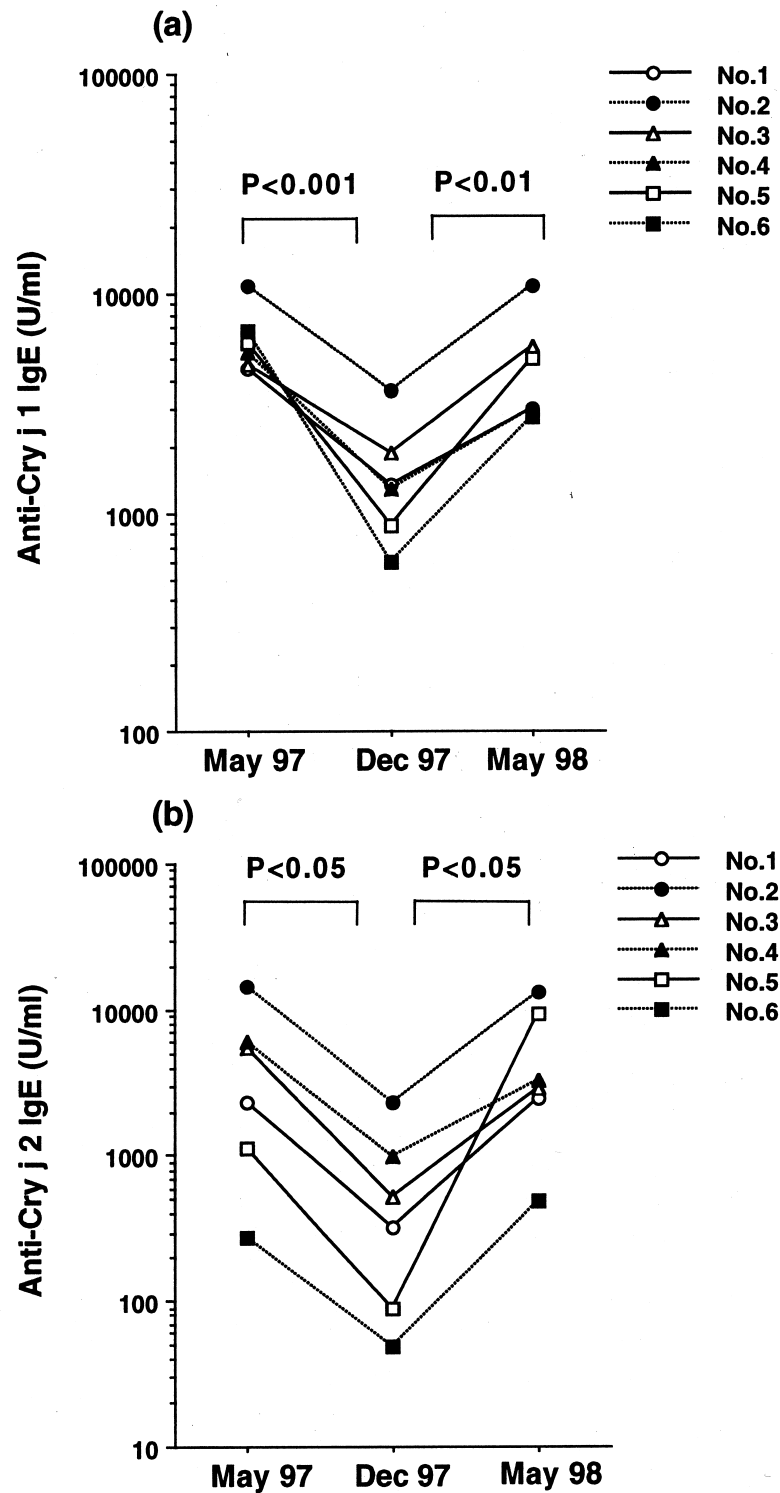


Fig. 2. Seasonal changes in specific IgE to (a) Cry j 1 and (b) Cry j 2 in the monkeys with CJ pollinosis.

crude CJ allergens showed a significant decline when compared with those in May 1997 ($P < 0.005$). Next, the levels of specific IgE to crude CJ allergens showed a significant increase after the pollen season in May 1998 when compared with those of December 1997 ($P < 0.01$).

Seasonal changes in specific IgE levels to Cry j 1 and Cry j 2

Figure 2a shows changes in specific IgE levels to Cry j 1 in these monkeys before and after CJ pollen season. After CJ pollen season in May 1997,

all monkeys had specific IgE to Cry j 1. Before CJ pollen season (December 1997), the levels of specific IgE to Cry j showed a significant decline when compared with those of May 1997 ($P < 0.001$). Next, the levels of specific IgE to Cry j 1 showed a significant increase after the pollen season when compared with those of December 1997 ($P < 0.01$).

Figure 2b shows seasonal changes in specific IgE levels to Cry j 2 in these monkeys before and after CJ pollen season. The levels of specific IgE to Cry j 2 showed significant seasonal changes similar to those of specific IgE to Cry j 1.

As a control, two monkeys without CJ pollinosis had no specific IgE to Cry j 1 and Cry j 2 in May 1997 (data not shown).

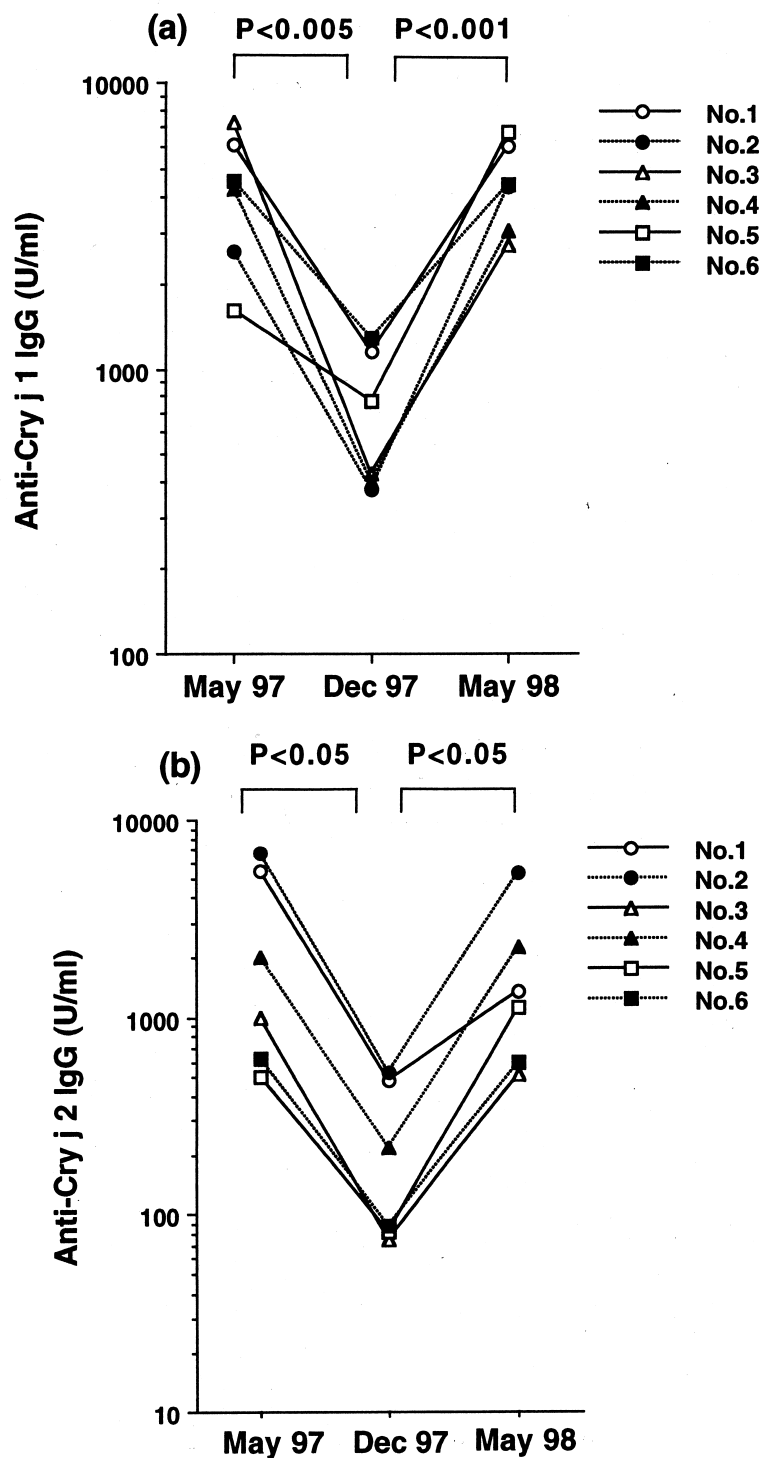


Fig. 3. Seasonal changes in specific IgG to (a) Cry j 1 and (b) Cry j 2 in the monkeys with CJ pollinosis.

Seasonal changes in specific IgG levels to Cry j 1 and Cry j 2

Figure 3a shows seasonal changes in specific IgG levels to Cry j 1 in these monkeys before and after CJ pollen season. After CJ pollen season (May 1997), all monkeys had specific IgG to Cry j 1. Before CJ pollen season (December 1997), the levels of specific IgG to Cry j 1 showed a significant decline when compared with those of May 1997 ($P > 0.005$). Next, the levels of specific IgG to Cry j 1 showed a significant increase after the pollen season when compared with those of December 1997 ($P > 0.001$).

Figure 3b shows seasonal changes in specific IgG levels to Cry j 2 in these monkeys before and after CJ pollen season. The levels of specific IgG to Cry j 2 showed significant seasonal changes similar to those of specific IgG to Cry j 1.

As a control, two monkeys without CJ pollinosis had no specific IgG to Cry j 1 and Cry j 2 in May 1997 (data not shown).

Seasonal changes in PBMC proliferative responses to major allergens

Table 1 shows original data of seasonal changes of PBMC proliferative response to Cry j 1 and Cry j 2 in these monkeys (nos. 1–6) before and after CJ

pollen season. As a control, two monkeys (nos. 7 and 8) without CJ pollinosis showed no PBMC proliferative response to Cry j 1 and Cry j 2 in May 1997.

Figure 4a shows seasonal changes in PBMC proliferative responses to Cry j 1 in these monkeys before and after CJ pollen season. After CJ pollen season (May 1997), all the monkeys showed PBMC proliferative response to Cry j 1. Before CJ pollen season (December 1997), PBMC proliferative response to Cry j 1 showed a significant decline compared with those of May 1997 ($P > 0.005$). Four monkeys (nos. 2–4 and 6) showed no specific PBMC proliferative response ($SI < 2.0$) before CJ pollen season. PBMC proliferative response to Cry j 1 showed a significant increase after the pollen season when compared with those of December 1997 ($P > 0.01$). However, SI in monkey no. 1 showed a decline when compared with that of December 1997. In monkey no. 1, a mean CPM in absence of antigen (background) was very high, so the SI was very low (Table 1).

Figure 4b shows seasonal changes of PBMC proliferative response to Cry j 2 in these monkeys before and after CJ pollen season. The levels of PBMC proliferative response to Cry j 2 showed significant seasonal changes similar to those of PBMC proliferative response to Cry j 1.

Table 1. Summary of seasonal changes in PBMC proliferative response to Cry j 1 and Cry j 2

Monkey	Allergen	May 1997			December 1997			May 1998		
		Allergen			Allergen			Allergen		
		+	–	SI	+	–	SI	+	–	SI
No. 1	Cry j 1	5,500	1,146	4.8	1,823	696	2.6	17,670	7,977	2.2
	Cry j 2	8,345	1,146	7.3	1,138	696	1.6	32,259	7,977	4.0
No. 2	Cry j 1	14,184	1,439	9.9	2,609	2,480	1.1	18,249	1,882	9.7
	Cry j 2	32,212	1,439	22.4	5,766	2,480	2.3	31,697	1,882	16.8
No. 3	Cry j 1	10,946	3,489	3.1	5,229	4,200	1.2	29,084	5,592	5.2
	Cry j 2	14,910	3,489	4.3	8,535	4,200	2.0	38,863	5,592	6.9
No. 4	Cry j 1	7,710	1,213	6.4	6,149	3,930	1.6	48,504	5,483	8.8
	Cry j 2	15,978	1,213	13.2	18,005	3,930	4.6	77,400	5,483	14.1
No. 5	Cry j 1	21,210	1,666	12.7	8,140	2,014	4.0	46,094	5,322	8.7
	Cry j 2	35,305	1,666	21.2	21,471	2,014	10.7	68,736	5,322	12.9
No. 6	Cry j 1	12,380	2,131	5.8	660	692	1.0	24,964	2,602	9.6
	Cry j 2	13,021	2,131	6.1	2,564	692	3.7	39,408	2,602	15.1
No. 7	Cry j 1	1,807	1,804	1.0	NT			NT		
	Cry j 2	1,715	1,804	0.95	NT			NT		
No. 8	Cry j 1	1,986	1,854	1.1	NT			NT		
	Cry j 2	2,412	1,854	1.3	NT			NT		

NT, not tested.

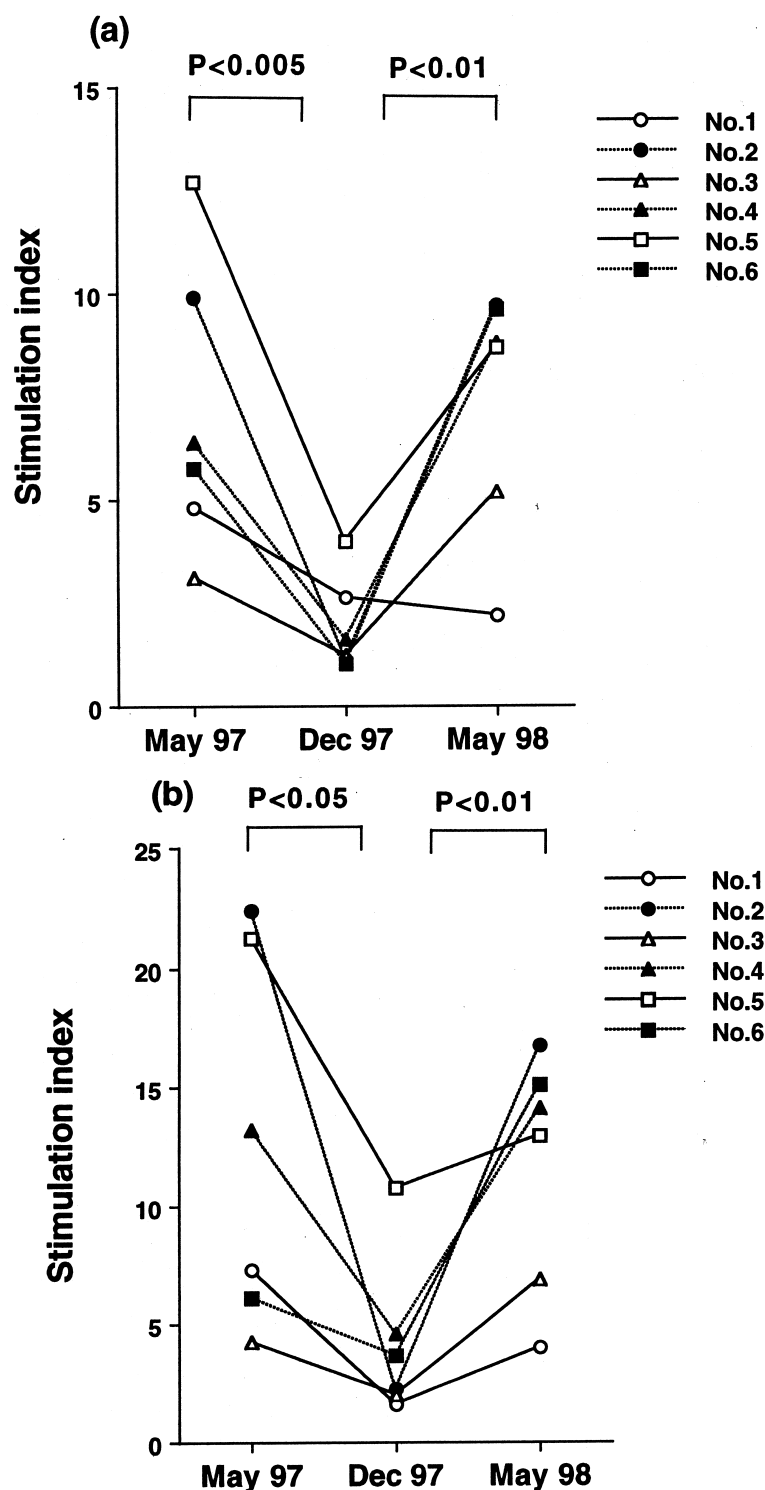


Fig. 4. Seasonal changes in PBMC proliferative response to (a) Cry j 1 and (b) Cry j 2 in the monkeys with CJ pollinosis.

Discussion

In this study, we examined seasonal changes in specific IgE levels to CJ allergens in monkeys with CJ pollinosis. Significant seasonal changes in specific IgE to crude CJ allergens were detected in the monkeys. Furthermore, we detected significant

changes in specific IgE to Cry j 1 and Cry j 2 in these monkeys. In human patients with CJ pollinosis, similar seasonal changes of specific IgE to crude CJ allergens and Cry j 1 and Cry j 2 have been reported [7, 10]. Decreases in the levels of anti-Cry j 1 and anti-Cry j 2 IgE responses in humans were detected before the pollen season.

Increases in the levels of specific IgE responses were detected after the pollen season. We confirmed that the levels of specific IgE to CJ allergens showed similar seasonal patterns to those of human patients.

There is no report discussing the levels of specific IgG to allergens in monkeys with allergic diseases. One goal of the present study was to examine whether or not the levels of specific IgG to Cry j 1 and Cry j 2 in monkeys with CJ pollinosis also showed seasonal change. In this study, we detected seasonal changes of specific IgG to Cry j 1 and Cry j 2 in the monkeys. In a previous study, we found that the levels of specific IgG to Cry j 1 in human patients with CJ pollinosis showed significant seasonal changes [10]. In humans, patterns of changes in the levels of specific IgG were similar to that of specific IgE. We confirmed that the levels of specific IgG to CJ allergens showed similar seasonal patterns to those of human patients. It has also been reported that the levels of allergen-specific IgG were elevated in sera of human patients with immunotherapy [3, 4, 18]. Measurement of specific IgG might serve as a biomarker that would assess possible immunotherapies for monkeys with CJ pollinosis.

We found seasonal changes in the PBMC proliferative response to Cry j 1 and Cry j 2 in these monkeys. In previous studies, we reported that PBMCs from monkeys during the pollen season showed proliferation responses to Cry j 1 and Cry j 2 [8, 23]. In the present study, all monkeys showed a specific PBMC proliferative response to CJ allergens after the pollen season. However, some of the monkeys showed no specific PBMC proliferative response to CJ allergens before the pollen season.

In the monkeys with CJ pollinosis, humoral and cellular immune responses to CJ allergens showed significant seasonal changes similar to those shown by human patients. Analysis of humoral and cellular immune responses might serve as biomarkers for assessing potential immunotherapies for pollinosis. Recently, we developed new immunotherapies for CJ pollinosis in mice [9, 28]. In a previous study, we found that oral immunotherapy with T-cell epitope peptide of Cry j 2 induces immunological tolerance in both TH1 and TH2 cell responses against the whole protein allergen [9]. Furthermore, we found that in mice plasmid DNA encoding Cry j 1 gene induces Cry j 1-specific TH1 immune responses, resulting in inhibition of the IgE response to Cry j 1 [28]. This study will provide baseline data for the evaluation of these new immunotherapies for monkeys with CJ pollinosis.

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