

IgE reactivity and cross-reactivity to Japanese cedar (*Cryptomeria japonica*) and cypress (*Chamaecyparis obtusa*) pollen allergens in dogs with atopic dermatitis

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Abstract

The natural occurrence of Japanese cedar (*Cryptomeria japonica*) pollinosis has been reported in dogs with atopic dermatitis. However, the reactivity to Japanese cypress (*Chamaecyparis obtusa*) pollen allergens in these dogs has not been reported. The present study was designed to investigate the reactivity to Japanese cypress pollen allergens in dogs sensitized to Japanese cedar pollen allergens. In 19 dogs with specific IgE to *C. japonica* pollen allergen, we measured the specific IgE to *C. obtusa* pollen allergen and examined the reactivity to the allergen by intradermal test. Of the 19 dogs, 18 had specific IgE to crude and purified major allergens (Cha o 1) of *C. obtusa* pollen. Most of the dogs showed a positive reaction to *C. obtusa* pollen allergens in the intradermal test. Allergic cross-reactivity between Cha o 1 and Cry j 1 (a major allergen in *C. japonica* pollen) was observed by the ELISA inhibition method. Dogs sensitized to Japanese cedar pollen allergens demonstrate reactivity to Japanese cypress pollen allergens. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Atopic dermatitis; Dog; IgE; Cedar pollen; Cypress pollen

Abbreviations: *C. japonica*, *Cryptomeria japonica*; *C. obtusa*, *Chamaecyparis obtusa*; FU, fluorescence units; IDST, intradermal skin test

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1. Introduction

Canine atopic dermatitis is a common disease in veterinary dermatology (Halliwell and Schwartzmann, 1971; Nesbitt et al., 1984; Willemse, 1986). Various clinical signs have been reported, including pruritus, otitis externa, bilateral conjunctivitis, and recurrent and chronic dermatitis (Willemse, 1986).

In humans, Japanese cedar (*Cryptomeria japonica*) pollinosis is one of the most common allergic diseases in Japan (Ishizaki et al., 1987). *C. japonica* pollinosis has also been reported in dogs in Japan (Sasaki et al., 1995). Our recent epidemiological study reported that approximately 20% of atopic dogs had specific IgE to crude *C. japonica* pollen allergen (Masuda et al., 2000a). Furthermore, dogs sensitive to *C. japonica* pollen allergen showed blastogenesis and histamine release from peripheral leukocytes specific to this pollen antigen (Masuda et al., 2000b). These findings suggested that *C. japonica* pollen allergen might be associated with the pathogenesis of atopic dermatitis in dogs.

In humans, Japanese cypress (*Chamaecyparis obtusa*) pollen also plays an important role in pollinosis during the spring season. Most human patients with *C. japonica* pollinosis have demonstrated reactivity to *C. obtusa* pollen allergen, based on skin tests and detection for allergen-specific IgE (Ito et al., 1995; Nohara et al., 1996). Furthermore, cross-allergenicity has been reported between *C. japonica* and *C. obtusa* pollen allergens (Panzani et al., 1986).

Two major allergens have been isolated from *C. japonica* pollen and characterized as Cry j 1 (Yasueda et al., 1983) and Cry j 2 (Sakaguchi et al., 1990). We previously showed that human patients sensitive to *C. japonica* pollen had specific IgE to Cry j 1 and/or Cry j 2 (Sakaguchi et al., 1990; Hashimoto et al., 1995). Recently, we found that out of 27 atopic dogs sensitive to *C. japonica* pollen, all had anti-Cry j 1 IgE, and 10 (37%) had anti-Cry j 2 IgE (Masuda et al., 2000c). This finding suggests that Cry j 1 is a major allergen in dogs. A major allergen was isolated from *C. obtusa* pollen, and has been designated Cha o 1 according to WHO nomenclature (Ide et al., 1995). The primary structure of Cha o 1 was determined by cDNA sequencing, and the amino acid sequence of Cha o 1 demonstrated 80% identity with that of the Cry j 1 (Suzuki et al., 1996).

The present study investigates the reactivity to *C. obtusa* pollen allergen in dogs sensitive to *C. japonica* pollen allergen. We measured the specific IgE to *C. obtusa* pollen allergen and examined the reactivity to the allergen by the intradermal test. Furthermore, we analyzed the cross-reactivity between major *C. japonica* and *C. obtusa* pollen allergens by the ELISA inhibition method.

2. Materials and methods

2.1. Crude and major *C. japonica* and *C. obtusa* pollen allergens

Pollen crude extract was prepared as previously described (Yasueda et al., 1983). Cry j 1 was purified from *C. japonica* pollen as previously described (Yasueda et al., 1983). Cha o 1 was purified from *C. obtusa* pollen according to the procedure of Ide et al., 1995. Briefly, 40 g of pollen was extracted with 1000 ml of 0.1 M Tris–HCl (pH 8) by shaking the sample

gently at 4°C for 16 h. The sample was centrifuged at $12000 \times g$ for 15 min, and the supernatant was collected. Next, the extracts were applied to a QAE-Toyopearl 550C column (Tosoh, Tokyo, Japan) equilibrated with 0.1 M Tris-HCl (pH 8) and the pass-through fraction was collected. The pass-through fraction was dialyzed against 0.1 M phosphate buffer (pH 5) and applied to a SP-Toyopearl 550C column (Tosoh) equilibrated with 0.1 M acetate buffer (pH 5), and the proteins were eluted with 0.2 M phosphate buffer (pH 7). The eluted material was applied to Sephacryl S-300 (Pharmacia, Uppsala, Sweden) equilibrated with phosphate-buffered saline. The allergenic activity of the fractions at the peak of the gel filtration was verified by a fluorometric ELISA for dog allergen-specific IgE.

2.2. Dogs

Nineteen dogs showed IgE sensitivity to crude *C. japonica* pollen allergen by the fluorometric ELISA. These dogs were diagnosed with atopic dermatitis according to the established criteria (Willemse, 1986), and supplied by the Veterinary Medical Center, University of Tokyo, Japan. They consisted of 13 females and 6 males, and ranged from 1- to 10-year-old (age $\pm$ S.D., 5 years and 7 months $\pm$ 3 years and 1 month). All 19 dogs showed typical clinical signs of atopic dermatitis, including pruritus, recurrent and chronic dermatitis, otitis externa, and superficial staphylococcal pyoderma. Cases of dermatomycosis and ectoparasite infection were excluded.

The sera used as a negative control in the pollen-specific IgE assay were obtained from 10 dogs in the USA, where there is no growth of *C. japonica* or *C. obtusa*.

2.3. Intradermal skin test (IDST)

IDST was performed according to the instructions supplied by the Greer Laboratories, Lenoir, NC, USA. Briefly, the pollen extract that we prepared (Yasueda et al., 1983) was diluted to a concentration of 1:20 or 1:50 with diluent containing 0.9% sodium chloride and 0.4% phenol. The diluent also was used as a negative control. A histamine solution (0.0275 mg/ml) was used as a positive control. These solutions of the extract and the controls were injected intradermally into clipped skin of the ventro-lateral thorax at a volume of 0.05 ml, using a syringe with a 26-gauge needle. The diameter of each wheal was measured 15–20 min after the injection. Each wheal was then classified into one of four sizes. A size equal to or greater than that of the positive control was +++. If the size was equal to or smaller than that of the negative controls, it was determined as negative (–). The mean size between the positive and negative controls was used to divide ++ and +; if a size was equal to or greater than the mean size, it was ++. The size smaller than the mean size was +.

2.4. Fluorometric ELISA for specific IgE to *C. japonica* and *C. obtusa* pollen allergens

Specific IgE to crude and major *C. japonica* and *C. obtusa* pollen allergens were assayed by a fluorometric ELISA with a slight modification from the method previously described (Sakaguchi et al., 1997). A microplate (Immulon 2, Dynatech, Chantilly, VA, USA) was coated with each of the crude *C. japonica* and *C. obtusa* pollen allergens (10 μ g/ml) or each of the purified allergens, Cry j 1 and Cha o 1 (1 μ g/ml) at 4°C overnight. For each allergen,

after washing, the plate was incubated with several different concentrations of serum samples at room temperature for 3 h. The plate was then washed and incubated with a mouse monoclonal anti-dog IgE antibody (0.5 µg/ml) (De Boer et al., 1993). The plate was incubated at 4°C overnight. After washing, the plate was next incubated with biotinylated rat monoclonal anti-mouse IgG (Zymed Laboratories) at room temperature for 1 h. After washing, the plate was incubated with β-D-galactosidase conjugated streptavidin (Zymed Laboratories, San Francisco, CA) at room temperature for 1 h. After final washing, the plate was incubated with 0.1 mM 4-methylumbelliferyl-β-D-galactoside (Sigma, St. Louis, MO, USA) at 37°C for 2 h. The enzyme reaction was stopped with 0.1 M glycine-NaOH (pH 10.2), and the fluorescence intensity was read as fluorescence units (FU) on a microplate fluorescence reader (Fluoroskan, Flow Laboratories, McLean, VA, USA). The level of specific IgE to each *C. japonica* and *C. obtusa* pollen allergen was calculated from a standard titration curve of a reference standard (pooled serum) and was expressed in arbitrary units (U/ml).

2.5. ELISA inhibition test

The allergenic cross-reactivity between *C. japonica* and *C. obtusa* pollen allergens was analyzed by a fluorometric ELISA inhibition. Each purified allergen, Cry j 1 and Cha o 1 (0.2 µg/ml) was adsorbed to microplate wells. After an overnight incubation at 4°C, the serum was mixed with Cry j 1 or Cha o 1 (20 µg/ml) as an inhibitor and added to the well. After 3 h incubation at room temperature, the plate was incubated with the anti-dog IgE antibody (0.5 µg/ml). The following procedures were the same as those in the above-described fluorometric ELISA. The percentage of inhibition was calculated as follows:

$$\text{inhibition (\%)} = \left[1 - \left(\frac{\text{FU in presence of allergen}}{\text{FU in absence of allergen}} \right) \right] \times 100$$

3. Results

3.1. IgE reactivity to crude *C. japonica* and *C. obtusa* pollen allergen in atopic dogs

Specific IgE to crude *C. japonica* and *C. obtusa* pollen allergens were measured in the sera of the 19 atopic dogs by the fluorometric ELISA (Fig. 1). Of the 19 dogs that had specific IgE to crude *C. japonica* pollen allergen, 18 had specific IgE to crude *C. obtusa* pollen allergen. The 10 dogs from the USA, used as negative controls had no specific IgE to crude *C. japonica* or *C. obtusa* pollen allergen (data not shown).

3.2. IgE reactivity to Cry j 1 and Cha o 1 in atopic dogs

IgE reactivity to Cry j 1 and Cha o 1 was examined by the ELISA in the 19 dogs sensitive to crude *C. japonica* pollen allergen (Fig. 2). Of the 19 atopic dogs, 18 had anti-Cha o 1 IgE. The 10 negative control dogs had no specific IgE to Cry j 1 or Cha o 1 (data not shown).

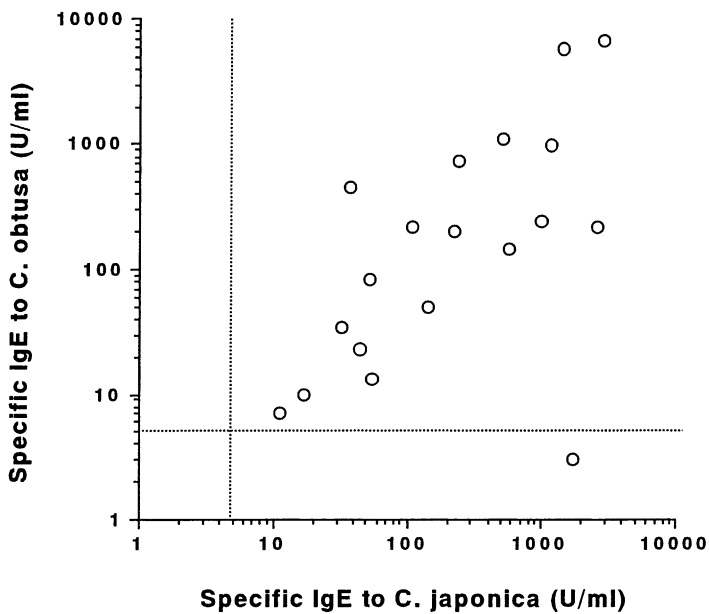


Fig. 1. IgE reactivity to crude *C. japonica* and *C. obtusa* pollen allergens in dogs.

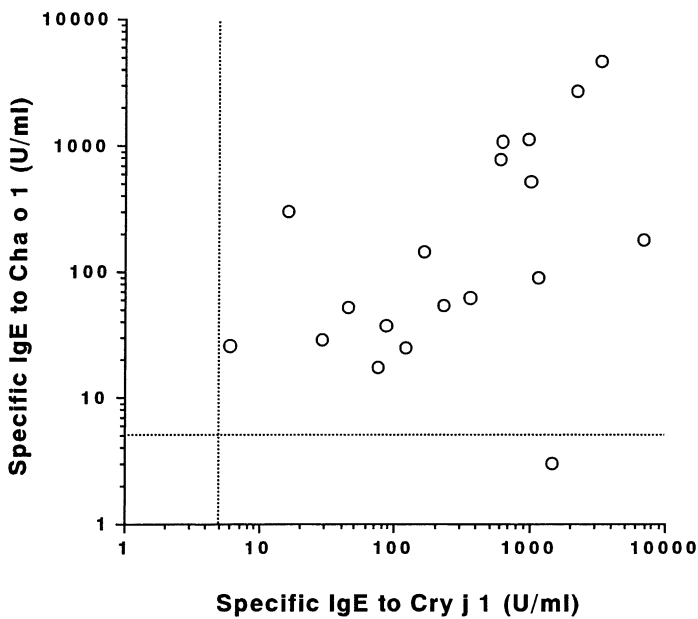


Fig. 2. IgE reactivity to *Cry j 1* and *Cha o 1* in dogs.

3.3. IDST reactions to crude and major *C. japonica* and *C. obtusa* pollen allergens in atopic dogs

In five atopic dogs sensitized to *C. japonica* pollen allergens, IDST tests were carried out (Table 1). All the five dogs had IDST reaction to crude *C. obtusa* pollen allergen and four had IDST reaction to Cha o 1. Comparison of IDST and ELISA results revealed that out of the five dogs with specific IgE to the crude *C. japonica* allergen by ELISA, all showed

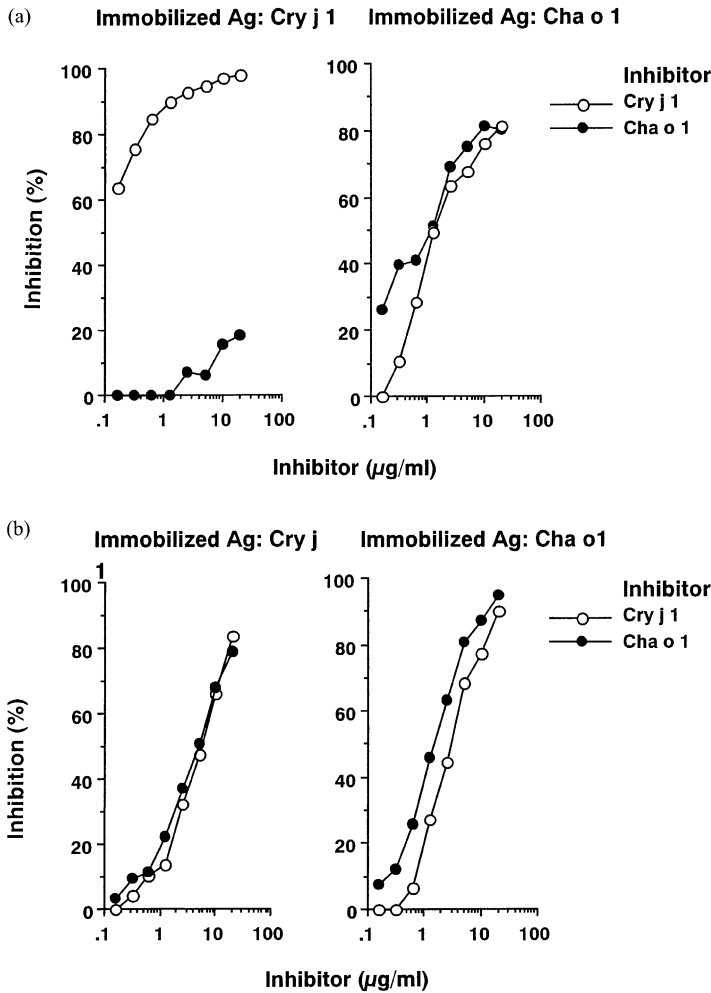


Fig. 3. Allergenic cross-reactivity between Cry j 1 and Cha o 1 by the ELISA inhibition. Each Cry j 1 and Cha o 1 was immobilized to microplate wells (a, b). The serum was mixed with Cry j 1 or Cha o 1 as an inhibitor and added to the well. ELISA inhibition experiments were performed in 10 dogs that have both anti-Cry j 1 and Cha o 1 IgE. In other dogs, the experiment could not be performed, since there was no sufficient serum quantity or the level of the specific IgE was low.

Table 1
Reactivity to *C. japonica* and *C. obtusa* pollen allergens in dogs

Dog	IDST				ELISA (U/ml)			
	<i>C. japonica</i>		<i>C. obtusa</i>		<i>C. japonica</i>		<i>C. obtusa</i>	
	Crude	Cry j 1	Crude	Cha o 1	Crude	Cry j 1	Crude	Cha o 1
1	+++	+++	+++	+++	33	85	34	37
2	+++	+++	+++	+++	143	234	50	54
3	+++	+++	+++	+++	226	994	204	517
4	+++	+++	+++	+++	240	165	738	142
5	+++	+++	+	–	2560	6887	217	182

positive IDST for the crude *C. obtusa* pollen allergen (Table 1). However, of the five dogs with specific IgE to Cha o 1, one showed no positive IDST for Cha o 1.

3.4. Cross-reactivity between Cry j 1 and Cha o 1 by ELSIA inhibition

In 10 dogs with both anti-Cry j 1 and Cha o 1 IgE, allergenic cross-reactivity between Cry j 1 and Cha o 1 was investigated by the ELISA inhibition. In seven dogs, incubation of the homologous Cry j 1 or Cha o 1 with serum, inhibited binding by more than 80%. IgE binding to the solid-phase Cha o 1 was greatly inhibited by Cry j 1, but IgE binding to the solid-phase Cry j 1 was not greatly inhibited by Cha o 1 (data not shown).

In other three dogs, incubation of the homologous Cry j 1 or Cha o 1 with serum, inhibited binding by more than 80%. IgE binding to the solid-phase Cha o 1 was greatly inhibited by Cry j 1. Similarly, IgE binding to the solid-phase Cry j 1 was greatly inhibited by Cha o 1 (data not shown). Fig. 3a and b showed typical dose-dependent ELISA inhibition curves in a dog. We found two patterns of cross-reactivity between Cry j 1 and Cha o 1, and Cry j 1 trends to show a greater inhibition than Cha o 1.

4. Discussion

Most of the dogs in this study, showed specific IgE to *C. obtusa* pollen allergen. Furthermore, dogs that demonstrated IgE to crude *C. japonica* pollen allergens showed positive reactions in the skin test after injection of crude *C. obtusa* pollen allergen. These findings are similar to those reported in human patients with pollinosis (Ito et al., 1995, 1996; Ide et al., 1995). Similarly, we found that monkeys sensitized to *C. japonica* pollen allergen also demonstrated reactivity to *C. obtusa* pollen allergens (Kobayashi et al., 1999). Dogs sensitized to *C. japonica* pollen allergen also demonstrated reactivity to *C. obsuta* pollen allergen.

Most of the human patients and monkeys with anti-Cry j 1 IgE had specific IgE to Cha o 1 (Ide et al., 1995; Suzuki et al., 1996; Kobayashi et al., 1999). Similarly, most of the dogs in this study, that demonstrated specific IgE to Cry j 1, also demonstrated specific IgE to Cha o 1. Furthermore, most dogs with anti-Cha o 1 IgE showed positive reactions

in the skin test after injection of Cha o 1. However, out of the five dogs with specific IgE to Cha o 1, one showed no positive IDST for Cha o 1. The reason for this discrepancy between the results of IDST and ELISA is as yet unknown. These findings suggest that, as in human patients and monkeys, Cha o 1 is an important allergen in dogs with pollinosis.

In ELISA inhibition, we found that there is allergenic cross-reactivity between Cry j 1 and Cha o 1, and Cry j 1 showed a greater inhibition than Cha o 1. In the Tokyo area during the spring season, the level of airborne *C. japonica* pollen is several times that of the airborne *C. obtusa* pollen (Nagano et al., 1992). It was reported that in the human patients specific IgE levels to *C. japonica* pollen allergen were higher than those to *C. obtusa* pollen allergen (Ito et al., 1995). From these findings, sensitization to *C. japonica* pollen allergen might be predominant in the humans and dogs. However, we think that it is difficult to find the primary sensitizing pollen allergen, because the pollen season for *C. japonica* overlaps with that of *C. obtusa*.

Recently, a second major allergen, Cha o 2, has been isolated from *C. obtusa* pollen (Yasueda et al., 2000). Also, it has been found that most of the human patients with pollinosis who had specific IgE to Cry j 2 showed IgE reactivity to Cha o 2 (Yasueda et al., 2000). In the future, we plan to analyze reactivity to Cha o 2 in dogs with pollinosis that have sensitivity to Cry j 2.

In conclusion, the dogs demonstrated specific IgE to the crude and purified major allergens (Cha o 1) of *C. obtusa* pollen. The dogs showed positive IDST reactions to *C. obtusa* pollen allergens. Based on the present findings, dogs sensitized to Japanese cedar pollen allergens appear to demonstrate reactivity to Japanese cypress pollen allergens.

References

- De Boer, D.J., Ewing, K.M., Schultz, K.T., 1993. Production and characterization of mouse monoclonal antibodies directed against canine IgE and IgG. *Vet. Immunol. Immunopathol.* 37, 183–199.
- Halliwell, R.E.W., Schwartzmann, R.M., 1971. Atopic disease in the dog. *Vet. Rec.* 89, 209–214.
- Hashimoto, M., Nigi, H., Sakaguchi, M., Inouye, S., Imaoka, K., Miyazawa, H., Taniguchi, Y., Kurimoto, M., Yasueda, H., Ogawa, T., 1995. Sensitivity to major allergens (Cry j 1 and Cry j 2) in patients with Japanese cedar (*Cryptomeria japonica*) pollinosis. *Clin. Exp. Allergy* 25, 848–852.
- Ide, T., Yamamoto, K., Tabata, S., Ashida, T., 1995. N-terminal amino acid sequence of a major allergen, Cha o 1, of Japanese cypress pollen. *Jpn. J. Palynol.* 41, 69–72 (in Japanese with English summary).
- Ishizaki, T., Koizumi, K., Ikemori, R., Ishiyama, Y., Kushibiki, E., 1987. Studies of prevalence of Japanese cedar pollinosis among the residents in a densely cultivated area. *Ann. Allergy* 58, 265–270.
- Ito, H., Nishimura, J., Suzuki, M., Mamiya, S., Sato, K., Takagi, I., Baba, S., 1995. Specific IgE to Japanese cypress (*Chamaecyparis obtusa*) in patients with nasal allergy. *Ann. Allergy Asth. Immunol.* 74, 299–303.
- Ito, H., Suzuki, M., Mamiya, S., Takagi, I., Baba, S., Tomita, K., Tanaka, A., 1996. Components of Hinoki cypress with the sera from patients with Japanese cedar pollinosis. *Allergol. Int.* 45, 181–186.
- Kobayashi, C., Nigi, H., Saito, S., Ide, T., Taniguchi, Y., Inouye, S., Sakaguchi, M., 1999. IgE reactivity and cross-reactivity of Japanese monkeys (*Macaca fuscata*) to Japanese cedar (*Cryptomeria japonica*) and cypress (*Chamaecyparis obtusa*) pollen allergens. *Clin. Exp. Allergy* 29, 856–861.
- Masuda, K., Sakaguchi, M., Fujiwara, S., Kurata, K., Yamashita, K., Odagiri, T., Nakao, Y., Matsuki, N., Ono, K., Watari, T., Hasegawa, A., Tsujimoto, H., 2000a. Positive reactions to common allergens in 42 atopic dogs in Japan. *Vet. Immunol. Immunopathol.* 73, 193–204.
- Masuda, K., Sakaguchi, M., Saito, S., DeBoer, D.J., Fujiwara, S., Kurata, K., Yamashita, K., a, A., Ohno, K., Tsujimoto, H., 2000b. In vivo and in vitro tests showing sensitization to Japanese cedar (*Cryptomeria japonica*) pollen allergen in atopic dogs. *J. Vet. Med. Sci.* 62, 995–1000.

- Masuda, K., Tsujimoto, H., Fujiwara, S., Kurata, K., Hasegawa, A., Taniguchi, Y., Yamashita, K., Yasueda, H., DeBoer, D.J., de Weck, A.L., Sakaguchi, M., 2000c. IgE reactivity to major Japanese cedar (*Cryptomeria japonica*) pollen allergens (Cry j 1 and Cry j 2) by ELISA in dogs with atopic dermatitis. *Vet. Immunol. Immunopathol.* 74, 263–270.
- Nagano, H., Nishima, S., Kishikawa, R., Sahashi, K., Yokoyama, T., 1992. Airborne Pollen in Japan, 2nd Edition. Hokuryukan, Tokyo, pp. 33–36 (in Japanese).
- Nesbitt, G.H., Kedam, G.S., Caciolo, R., 1984. Canine atopy. Part I. Etiology and diagnosis. *Compend. Contin. Educ. Vet.* 6, 73–84.
- Nohara, O., Imai, T., Saneyoshi, K., Endo, A., Nagakura, H., Ono, M., Moriyama, H., 1996. Comparative study on the assay for IgE antibodies specific for *Chamaecyparis obtusa* pollen between AlaSTAT and CAP-RAST. *Jpn. J. Allergol.* 45, 562–569.
- Panzani, R., Yasueda, H., Shimizu, T., Shida, T., 1986. Cross-reactivity between the pollens of *Cupressus sempervirens* (common cypress) and of *Cryptomeria japonica* (Japanese cedar). *Ann. Allergy* 57, 36–40.
- Sakaguchi, M., Inouye, S., Taniai, M., Ando, S., Usui, M., Matsuhashi, T., 1990. Identification of the second major allergen of Japanese cedar pollen. *Allergy* 45, 309–312.
- Sakaguchi, M., Nakano, T., Tsujimoto, H., Sasaki, Y., DeBoer, D.J., Inouye, S., 1997. Fluorometric ELISA for allergen-specific dog IgE antibody. *Allergol. Int.* 46, 207–212.
- Sasaki, Y., Kitagawa, H., Fujioka, T., Kitoh, K., Iwasaki, T., Sakaguchi, M., Inouye, S., 1995. Hypersensitivity to Japanese cedar (*Cryptomeria japonica*) pollen in dogs. *J. Vet. Med. Sci.* 57, 683–685.
- Suzuki, M., Komiyama, N., Itoh, M., Itoh, H., Sone, T., Kino, K., Takagi, I., Ohta, N., 1996. Purification, characterization and molecular cloning of Cha o 1, a major allergen of *Chamaecyparis obtusa* (Japanese cypress) pollen. *Mol. Immunol.* 33, 451–460.
- Willemse, T., 1986. Atopic skin disease: a review and a reconsideration of diagnostic criteria. *J. Small Anim. Pract.* 27, 771–778.
- Yasueda, H., Yui, Y., Shimazu, T., Shida, T., 1983. Isolation and partial characterization of the major allergen from Japanese cedar (*Cryptomeria japonica*) pollen. *J. Allergy Clin. Immunol.* 71, 77–86.
- Yasueda, H., Saito, A., Sakaguchi, M., Ide, T., Saito, S., Taniguchi, Y., Inouye, S., 2000. Identification and characterization of a group 2 conifer pollen allergen from *Chamaecyparis obtusa*, a homologue of Cry j 2 from *Cryptomeria japonica*. *Clin. Exp. Allergy* 30, 546–550.