***In vivo* and *In vitro* Tests Showing Sensitization to Japanese Cedar (*Cryptomeria japonica*) Pollen Allergen in Atopic Dogs**

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ABSTRACT. Using both *in vivo* and *in vitro* tests, dogs with atopic dermatitis were examined for sensitization with Japanese cedar (*Cryptomeria japonica*, CJ) pollen allergen. Ten dogs with clinical manifestation of atopic dermatitis were shown to be sensitized to CJ pollen based on the results of intradermal skin test and serum antigen-specific IgE test. *In vitro* lymphocyte stimulation test showed blastogenic response after stimulation with crude antigen of CJ pollen in all of the 5 cases examined. The peripheral leukocytes showed increased histamine release after stimulation with crude antigen of CJ pollen in 2 cases examined. These data indicate that a proportion of dogs with atopic dermatitis is sensitized to CJ pollen in a cell-mediated manner and show immediate phase reaction of type I hypersensitivity.

KEY WORDS: atopic dermatitis, canine, Japanese cedar pollen, type I hypersensitivity.

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Japanese cedar (*Cryptomeria japonica*, CJ) pollinosis in humans is one of common seasonal allergies in Japan, causing rhinitis and conjunctivitis as clinical symptoms [7, 20]. In CJ pollinosis, *in vivo* and *in vitro* evidences of immediate phase reaction of type I hypersensitivity to CJ pollen allergen have been reported, including positive results in intradermal skin test (IDST) [11], serum antigen-specific IgE test [7], and antigen-specific histamine release assay [5]. In contrast to these studies on CJ pollinosis in humans, CJ pollinosis has been rarely investigated in dogs, although a relatively high proportion of atopic dogs in Japan is known to be sensitized to CJ pollen allergen [10]. In terms of reactivities to CJ pollen allergen in dogs, only one study indicated that atopic dogs sensitized to CJ pollen allergen showed nasal discharges in a provocative challenge of CJ pollen allergen to the nasal mucosal membrane [17], but no *in vitro* study to show the sensitization to CJ pollen allergen has been carried out. *In vivo* and *in vitro* tests to show the reactivities to CJ pollen allergen are necessary to provide a basic information on the sensitization to CJ pollen allergen in dogs.

As *in vitro* tests to show the sensitization to allergens, tests for antigen-specific lymphocyte proliferation response [19] and antigen-specific histamine release [5, 8, 9, 14] have been employed to examine the responses to antigens in allergic diseases. In patients with allergic diseases, some populations of T-cells reacting to T-cell epitopes on the allergen exist in the peripheral blood, so that the lymphocyte proliferation responses to antigens have been considered as an *in vitro* diagnostic tool to identify the cell-mediated immune responses to allergens [19]. Basophils and mast cells express FcεRI on the cell surface and release histamine through cross-linking of IgE bound to the FcεRI after antigen stimulation [1, 8, 18]. Peripheral blood basophils have been used

for investigation of the histamine releasability in individuals with allergic diseases to indicate a direct *in vitro* evidence of type I hypersensitivity [5, 6, 8]. The assay of the histamine release was therefore considered to be applicable to show type I hypersensitivity in dogs.

In the present study, sensitization to CJ pollen allergen in dogs with atopic dermatitis was examined with both *in vivo* and *in vitro* tests including intradermal skin test, serum antigen-specific IgE test, lymphocyte stimulation test, and antigen-specific histamine release assay.

MATERIALS AND METHODS

Selection of dogs sensitized to CJ pollen allergen: Based on Willemse's criteria [22], clinical diagnosis of atopic dermatitis in dogs was performed for 42 dogs with pruritus at Veterinary Medical Center, the University of Tokyo after exclusion of other pruritic skin diseases caused by parasitic infestations (i.e. demodicosis, fleas, or scabies), cutaneous infections (i.e. dermatophytosis and bacterial pyoderma), and seborrhea. Food allergy was excluded in most instances from the history of the dermatologic conditions related to food intake, although tests using hypoallergenic diets in combination with challenge diets could not be carried out in all the cases. To identify sensitization to CJ pollen allergen, serum antigen-specific IgE test and intradermal skin test were carried out as follows.

Intradermal skin test: A crude extract of CJ pollen was prepared at a concentration of 10 µg/ml by a method previously reported [23]. The allergen extract was diluted with a diluent containing 0.9% sodium chloride and 0.4% phenol at a final concentration of 200 ng/ml. The diluent without allergen extracts was used as a negative control. Histamine solu-

tion diluted at a concentration of 0.0275 mg/ml was used as a positive control. These solutions (0.05 ml of each per test) were carefully injected intradermally into the clipped skin of the ventro-lateral thorax, by means of a skin test syringe attached with a 26-gauge needle. The diameters of wheals were measured 15 to 20 min after injection. The results of the intradermal skin test were graded as follows: +++, equal to or greater than the diameter of the positive control; ++, equal to or greater than the mean diameter of the positive and negative controls; +, larger than the diameter of the negative control but small than the mean diameter of the positive and negative controls; -, equal to or smaller than the diameter of the negative control.

Antigen-specific IgE test: IgE antibodies specific to CJ pollen allergens were assayed by a fluorometric ELISA as described previously [16]. A microplate (Immulon 2, Dynatech, Chantilly, VA) was coated with crude CJ pollen antigens (10 µg/ml) at 4°C overnight. After washing, diluted serum samples were added to the wells and incubated at room temperature for 3 hr. The plate was incubated with mouse monoclonal anti-dog IgE antibody [2] (0.5 µg/ml) at 4°C overnight and then biotinylated rat monoclonal anti-mouse IgG₁ (Zymed Laboratories, San Francisco, CA) at room temperature for 1 hr. The plate was further incubated with β-D-galactosidase conjugated streptavidin (Zymed Laboratories, San Francisco, CA) at room temperature for 1 hr, and 0.1 mM 4-methylumbelliferyl-β-D-galactoside (Sigma Chemical Co., MO) at 37°C for 2 hr. The enzyme reaction was stopped with 0.1 M glycine-NaOH (pH 10.2), and the fluorescence intensity was measured as fluorescence units (FU) on a microplate fluorescence reader (Fluoroskan, Flow Laboratories, McLeane, VA). The level of IgE specific to CJ pollen allergens was expressed in arbitrary units (U/ml), and calculated from a standard titration curve of a pooled serum.

Lymphocyte stimulation test: Heparinized peripheral blood samples were diluted with an equal volume of phosphate buffered saline (PBS) and layered on Ficoll-Hypaque (Nycomed Pharma AS, Oslo, Norway). After centrifugation at 350 g for 40 min at room temperature, a layer of peripheral blood mononuclear cell (PBMC) fraction was obtained and suspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% dog serum and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml) at a cell count of 1.25×10^6 /ml. The cell suspension (200 µl) allocated to each well of a 96-well plate was incubated at 37°C for 72 hr under stimulation of the crude extract of CJ pollen (3 µg/ml). Blastogenic responses of the antigen-stimulated and unstimulated groups were measured with the assay of incorporation of ³H-thymidine (1 µCi/well) for 18 hr by using triplicated cultures. Radioactivity of the incorporation was measured by liquid scintillation counter. Stimulation Index (SI) was expressed by the ratio of the mean incorporated radioactivity in the antigen-stimulated groups to that in unstimulated groups (antigen-stimulated groups/unstimulated groups).

Antigen-specific histamine release test: Heparinized peripheral blood samples were mixed with one fourth volume of PBS containing 5% dextran, and incubated at room

temperature for 60 min. The leukocyte fraction containing basophils was collected and washed with 0.03 M Tris-HCl containing 0.03% fetal bovine serum and 1 mM EDTA and then with the same buffer without EDTA, and finally suspended in DMEM at a cell count of $1-3 \times 10^7$ /ml. The percentage of basophils in the suspension was counted on a blood smear stained with Wright stain solution (Wako Pure Chemical Industries, Osaka, Japan). The leukocyte suspension (450 µl of each sample) was exposed to various concentrations (0.01–100 ng/ml) of crude CJ pollen extract at 37°C for 60 min under gentle shaking. As a control, the leukocyte suspension was incubated without exposure to antigens to assess the amount of naturally released histamine. The samples of the supernatants from these cultures were stored at –80°C for subsequent assay of the amount of released histamine. The total histamine content in 450 µl of the cell suspension was obtained after cell lysis with hypotonic shock with distilled water, freezing at –80°C, and thawing at 80°C. After centrifugation at 300 g for 5 min, the supernatant was stored at –80°C until assay for the histamine content.

The histamine content was measured by competitive radio-immunoassay with a histamine assay kit (Immunotech International, Marseilles, France) according to a method previously described [9]. The histamine content in the samples was obtained from the standard curve prepared from various concentrations (0–150 nM) of standard histamine. After the amount of histamine released without stimulation was subtracted from that released after stimulation, antigen-specific histamine release was expressed as a percentage of the total amount of histamine content in the leukocyte fraction.

RESULTS

Dogs sensitized to CJ pollen allergen: Of the 42 dogs with atopic dermatitis, 10 were found to be sensitized to CJ pollen allergen in this study (Table 1). They showed positive reactions to CJ pollen allergen in intradermal skin test and antigen-specific IgE test (Table 2). There was no apparent predisposition due to breed or sex. In 7 of the 10 cases, the first onset of clinical signs was recorded at less than 4 years of age. All the dogs had pruritus, but other clinical manifestations differed among the cases. Dogs with a long history of atopic dermatitis were prone to show secondary and chronic signs such as pustules, pigmentation, lichenification and hair loss (Cases 1, 2, 3 and 7). Apparent seasonality of the symptoms was detected in Cases 5, 6, 7, 9 and 10, all of which showed aggravation of the symptoms in spring from March to May. In the rest of the cases, the clinical signs were consistently seen throughout the year.

Intradermal skin test: The results of intradermal skin test and antigen-specific IgE test are shown in Table 2. In these 10 dogs, reactive wheals were observed 15 to 20 min after injection of CJ pollen allergen extract in the intradermal skin test. In the 9 dogs (Cases 1 to 9), distinct wheals (recorded as +++) larger than the positive histamine control were observed. Only Case 10 had a slightly smaller wheal

Table 1. Signalment and clinical signs in dogs sensitized to CJ pollen allergen

No.	Breeds	Age (yrs)	Sex	Age at the 1st onset (yrs)	Clinical signs						
					Pruritus	Papules	Pustules	Pigmentation	Lichenification	Hair loss	Otitis externa
1	West Highland white terrier	9	F	4	+	+	+	+	+	+	+
2	West Highland white terrier	6	C	2	+	+	+	+	+	+	+
3	Akita	6	F	4	+	+	+	+	+	+	-
4	Great Pyreneese	2	F	2	+	+	+	-	-	-	-
5	Miniature dachshund	4	M	1	+	-	-	-	-	-	-
6	Mongrel	7	M	3	+	+	+	+	+	+	+
7	Japanese terrier	1	F	1	+	+	+	-	-	-	+
8	Irish setter	3	F	2	+	-	-	-	-	-	-
9	Cavalier King Charles spaniel	8	M	1	+	-	-	-	-	-	-
10	Shih tzu	10	M	9	+	+	-	-	-	-	-

F: female, M: male, and C: castrated male.

Table 2. Results of IDST and antigen-specific IgE test for CJ pollen allergen

Case No.	IDST	Antigen-specific IgE (U/ml)
1	+++	240.0
2	+++	33.0
3	+++	17.2
4	+++	52.4
5	+++	54.6
6	+++	226.0
7	+++	522.0
8	+++	980.0
9	+++	750.0
10	++	3.4

(recorded as ++) in the intradermal skin test.

IgE specific to CJ pollen allergen: The level of IgE specific to CJ pollen allergen ranged from 3.4 to 980.0 U/ml in the 10 dogs (Mean; 346.9, SD; 346.82). The IgE levels were not correlated with the severity of clinical signs of atopic dermatitis in these cases. The level of IgE specific to CJ pollen allergen in Case 10, that had a weaker reaction than other cases in the IDST, was lower than in the other cases.

Lymphocyte blastogenic response: Lymphocyte stimulation test was performed in 5 (Cases 1, 2, 6, 8 and 10) of the 10 cases in this study. All of the 5 dogs showed lymphocyte blastogenic response after stimulation with CJ pollen crude extract (Fig.1). Various degrees of distinct blastogenic responses were seen in these 5 cases. The stimulation index (SI) after stimulation with crude CJ pollen extract (3 µg/ml) ranged from 4.0 to 6.5 in these 5 cases.

Antigen-specific histamine release: The amount of total histamine content in the peripheral leukocytes was 363.0 to 3174.6 pg per 10⁷ leukocytes in the 6 dogs sensitized to CJ pollen allergen (Cases 1, 2, 3, 6, 7 and 10). Only Case 3, which had relatively many basophils (0.2% of leukocytes) in the peripheral blood, had a high level of the total histamine content in the leukocyte fraction. In the other dogs, the number of basophils was less than 0.1% of the leukocytes.

Leukocyte samples from 6 dogs (Cases 1, 2, 3, 6, 7, and 10) were subjected to antigen-specific histamine release test, but in 4 (Cases 2, 6, 7, 10) of the dogs, since the amount of naturally released histamine was almost equal to the total his-

Fig. 1. Blastogenic responses of lymphocytes with and without stimulation with CJ pollen allergen. The proliferation responses are shown with the amount of ³H-thymidine (mean ± standard error) incorporated into the cells.

tamine content of the leukocyte fraction, antigen-specific histamine release could not be evaluated. By contrast, in Cases 1 and 3, the amount of naturally released histamine was low, and histamine release from the leukocyte fraction increased according to the concentrations of CJ pollen extract and the maximum values were seen at the antigen concentration of 10 ng/ml in both cases (Case 1: 24.1 %, Case 3: 86.2%) (Fig. 2).

DISCUSSION

Five of the 10 dogs sensitized to CJ pollen allergen in this study showed aggravation of clinical signs in spring (March to May) which is the season of CJ pollination, however, apparent seasonality of the skin lesion was not detected in the other 5 dogs. It has been observed that cross-reactivity among plant allergens [15] prolongs the symptoms after the season of CJ pollination, since IgE specific to one plant allergen may react with similar epitopes of other plant allergens [21]. In fact, our screening assay system for IDST-positive

Fig. 2. The amount of histamine release specific to CJ pollen allergen from the peripheral leukocyte fraction containing basophils.

allergens revealed that positive reactions to the other plant allergens such as common sagebrush, yellow dock, grass mix (Kentucky blue, orchard, red top, timothy, sweet vernal, meadow fescue and perennial rye), eastern oak mix (white ash, American beech, red birch, shagbark hickory, sugar maple, red oak, sweet gum and eastern sycamore), and birch white were detected in Cases 1, 2, 4, 7 and 8. In serum antigen-specific IgE test for the other plant allergens, IgE against grass mix (cocksfoot, meadow fescue, perennial rye, timothy, Kentucky blue grass, velvet grass and yorkshire), weed mix (mugwort and ribwort), and tree mix (birch, oak and hazel) was found in Cases 1, 4 and 7. Moreover, Cases 2 and 3 were shown to be reactive to house dust mite allergens in IDST and antigen-specific IgE test, which can cause persistent pruritus all the year around [3]. These factors conceivably obscure the seasonality of the symptoms of atopic dermatitis in these dogs sensitized to CJ pollen allergen.

In this study, the allergen extract of CJ pollen for IDST was prepared as reported by Yasueda *et al.* [23]. This allergen extract has been routinely used in our clinic for IDST [10]. We recognized that IDST with the allergen extract of CJ pollen might cause cross-reactions with other plant allergens, however, antigen-specific IgE test for CJ pollen allergen was relatively specific and did not show obvious cross-reaction with other plant allergen [10]. In addition, we performed IDST with the allergen extract of CJ pollen for a number of healthy dogs and found no positive reactions in these dogs (data not shown). Therefore, the allergen extract of CJ pollen used in this study can be used to examine the sensitization to CJ pollen allergen in dogs.

There still remains a controversy concerning the difference between the clinical signs in humans and dogs sensitized to CJ pollen allergen. Although the major clinical signs in human CJ pollinosis are rhinitis and conjunctivitis [9], the symptom in the dogs sensitized to CJ pollen allergen in this

study was atopic dermatitis. Sasaki *et al.* [17] reported that atopic dogs naturally sensitized to CJ pollen allergen showed nasal discharge in a provocation test with CJ pollen extract. This observation suggests that allergic reactions in the nasal cavity can occur in dogs sensitized to CJ pollen, but in dogs allergic reaction is more evident in the skin than in the nasal cavity due to some unknown factor in dogs. In humans, it was reported that stem cell factor produced by nasal epithelial cells was related to the focal accumulation, proliferation and activation of mast cells, causing allergic rhinitis [13]. It is conceivable that some similar factors activating mast cells may exist in the skin of dogs and contribute to the development of atopic dermatitis rather than rhinitis as a reaction of type I hypersensitivity. Further investigation will be necessary to clarify the difference between the clinical signs in humans and dogs sensitized to CJ pollen allergen.

The results of lymphocyte stimulation test indicated that there were lymphocytes showing blastogenesis after stimulation with CJ pollen allergen in the dogs sensitized to CJ pollen allergen. Since the blastogenic response of lymphocytes after stimulation with CJ pollen allergen has been shown in human CJ pollinosis [19], it is conceivable that sensitization to CJ pollen allergen in dogs can be established in a similar manner to that in humans and monkeys with CJ pollinosis. In lymphocyte stimulation test, T-cell epitopes of CJ pollen allergen have been detected in humans [19]. Likewise, further study will be needed for the identification of T-cell epitopes of CJ pollen allergen in dogs. Thus, dogs sensitized to CJ pollen allergen can be recognized as a useful animal model of human CJ pollinosis for the development of a new immunotherapy by using T-cell epitope peptides [19] or DNA vaccine [12].

The values for total histamine content of leukocytes in atopic dogs in this study were similar to those in normal dogs and dogs experimentally sensitized to *Ascaris* antigen [9,

14]. The amount of antigen-specific histamine release after exposure to CJ pollen extract in this study was greater than that after exposure to *Dermatophagoides farinae* allergen in atopic dogs, as previously reported [9]. Histamine release of basophils in non-atopic dogs experimentally sensitized to antigens was shown to be negligible or very low [9, 14]. Histamine release after exposure to CJ pollen allergen was seen at antigen concentrations of 1 and 10 ng/ml in the present study. This finding is consistent with a previous report on humans with CJ pollinosis from which peripheral leukocytes released histamine after stimulation with the major allergens of CJ pollen at the concentrations of 0.1 to 10.0 ng/ml [5]. Considering the similarity between humans and dogs in the optimal antigen concentrations which induced histamine release after exposure to CJ pollen allergen, there seems to be a common type I hypersensitivity reaction in both humans and dogs sensitized to CJ pollen allergen.

In this study, antigen-specific histamine release was clearly demonstrated in 2 of the 10 cases sensitized to CJ pollen allergen, however, the amount of naturally released histamine without any antigen stimulation was larger than or equal to the amount of histamine release under antigen stimulation in the other 8 cases. The antigen-specific histamine release test can be considerably influenced by experimental conditions. When the amount of naturally released histamine was large, it was difficult to estimate the amount of antigen-specific histamine release. Such unstable characteristics of basophils will produce technical problems in some cases.

From the data obtained in this study, a possible mechanism to develop immediate phase reaction of type I hypersensitivity was demonstrated in dogs sensitized to CJ pollen allergen. Positive reactions to CJ pollen allergen in IDST were *in vivo* evidence of immediate phase reaction of type I hypersensitivity [17]. An increased level of IgE against CJ pollen allergen implied that the dogs were maintained in an "atopic" condition, also seen in humans [5, 7] and monkeys [4] with CJ pollinosis. The evidences of antigen-specific histamine release in dogs indicated that histamine-mediated inflammatory response could be induced through antigen binding and cross-linking of IgE on mast cells and basophils. Furthermore, the results in the lymphocyte stimulation test indicated that there were T lymphocytes reactive to CJ pollen allergen in the peripheral blood, as reported in humans with CJ pollinosis [19], which might induce Th2 type immune response to produce IgE against CJ pollen allergen. Thus, all the findings in this study can be seen to agree with each other, showing that the immune system develops immediate phase reaction of type I hypersensitivity to CJ pollen allergen in dogs with atopic dermatitis. For further characterization of the immunological status, investigation on the cytokine profile in dogs will be necessary to understand the mechanisms involved in the development of type I hypersensitivity to CJ pollen allergen in dogs.

The data in this study only indicate that a proportion of atopic dogs in Japan are sensitized to CJ pollen. At this point, it is still unknown whether atopic dermatitis in dogs

can be induced by sensitization to CJ pollen allergen. A provocation test in atopic dogs sensitized to CJ pollen allergen induced the secretion of nasal discharge after the challenge of CJ pollen allergen to the nasal mucosa, but did not induce any change of the skin [17]. Further provocative challenge tests in atopic dogs sensitized to CJ pollen allergen should be designed to know its involvement in the pathogenesis of canine atopic dermatitis.

In conclusion, we identified immediate phase reactions of type I hypersensitivity to CJ pollen allergen in dogs with atopic dermatitis *in vivo* and *in vitro*. Further study will be necessary to find whether CJ pollen allergen can be one of the factors to induce atopic dermatitis.

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