

Identification of peptides containing T-cell epitopes of Japanese cedar (*Cryptomeria japonica*) pollen allergen (Cry j 1) in dogs

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Abstract

Japanese cedar (*Cryptomeria japonica*, CJ) pollen has been known to cause atopic dermatitis in dogs in Japan. However, since the mechanism of the CJ antigen recognition is not well understood in dogs, it is difficult to develop effective immunotherapy for atopic dermatitis caused by sensitization to CJ pollen. In order to aim at development of a peptide immunotherapy, we tried to identify T-cell epitopes of a major allergen of CJ pollen, Cry j 1, in dogs sensitive to CJ pollen allergen. Peripheral blood mononuclear cells (PBMCs) obtained from 22 dogs experimentally sensitized to CJ pollen allergen and 5 atopic dogs sensitive to CJ pollen allergen were used for mapping of T-cell epitopes of Cry j 1 using 35 kinds of synthesized overlapping peptides of Cry j 1. Reactive peptides were identified based on the results of blastogenic responses of PBMCs against the peptides when the stimulation indices were beyond 2.0. Three reactive peptides were identical in a relatively high population of experimental dogs, which were Nos. 8 (p71–90) (41%), 10 (p91–110) (50%), and 11 (p101–120) (41%). It was considered that these synthesized peptides should contain T-cell epitopes of Cry j 1 in the dogs. However, there were no reactive peptides identical among the five atopic dogs spontaneously sensitive to CJ pollen. The population of dogs experimentally sensitized to CJ pollen antigen will be used in order to investigate effects of a peptide immunotherapy using the reactive peptides. The results in atopic dogs sensitive to CJ pollen antigen will also provide useful information on necessity to develop a tailor-made immunotherapy using reactive peptides in each dog.

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Keywords: Japanese cedar (*Cryptomeria japonica*); Pollinosis; Cry j 1; T-cell epitopes; Atopic dermatitis; Dog; Overlapping peptides

1. Introduction

Japanese cedar (*Cryptomeria japonica*, CJ) pollen is known to cause allergic reactions in humans in Japan (Ishizaki et al., 1987). Clinical presentation

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of CJ pollinosis is characterized by rhinitis and conjunctivitis as a consequence of type I hypersensitivity to CJ pollen allergen (Hashimoto et al., 1995; Ozasa et al., 2002). Many studies have proved allergic reactions to CJ pollen allergen in patients with CJ pollinosis by various allergy tests such as intradermal testing, IgE testing (Hashimoto et al., 1995; Ozasa et al., 2002), histamine release (Hashimoto et al., 1995), and lymphocyte blastogenic response (Hashimoto et al., 1995; Sone et al., 1998). It is known that these allergic reactions are caused by two major allergens of CJ pollen, Cry j 1 and Cry j 2, which induce IgE production (Sakaguchi et al., 1997; Yasueda et al., 1983), lymphocyte blastogenic response (Hashimoto et al., 1995), and Th2-skewed cytokine production in humans (Nakai et al., 1998) in an antigen-specific manner.

In a majority of human patients with CJ pollinosis, it was found that the major allergens were recognized by T-cell receptor (TCR) and major histocompatibility complex (MHC) through their interaction with T-cell epitopes consisting of two peptides (p108–120 and p211–225) in Cry j 1 and three peptides (p182–200, p344–355, and p66–80) in Cry j 2 (Sone et al., 1998). Hybrid peptides containing seven kinds of T-cell epitopes of Cry j 1 and Cry j 2 were predominantly recognized by peripheral lymphocytes from patients with CJ pollinosis, implying that an effective induction of T-cell tolerance might be possibly induced by the peptides (Hirahara et al., 2001). Since the peptides containing T-cell epitopes are recognized by antigen-specific T-cells but do not bind to antigen-specific IgE, anaphylactic side effects will not be provoked by a peptide immunotherapy using the peptides (Hirahara et al., 2001). Injections of major T-cell epitope peptides were shown to improve the clinical manifestations of allergic symptoms in patients with allergies to cat epithelia (Norman et al., 1996) and honey bee venom (Muller et al., 1998). Thus, identification of the peptides recognized by T-cells would be a useful, important approach for developing immunotherapy for allergic diseases.

In dogs, spontaneous sensitization to the antigen of CJ pollen and clinical symptoms associated with the sensitization to CJ pollen have been reported (Sasaki et al., 1995). It has been shown that approximately 10% of dog population with atopic dermatitis in Japan were sensitive to CJ pollen allergen, as shown by

intradermal testing, antigen-specific IgE testing (Masuda et al., 2000a), histamine release, and lymphocyte blastogenic response against CJ pollen antigen (Masuda et al., 2000b). It was shown that all the atopic dogs with increased levels of serum IgE specific to CJ pollen allergen had IgE against Cry j 1; however, only 37% of the dogs had Cry j 2-specific IgE (Masuda et al., 2000c), suggesting that Cry j 1 appears to more important for inducing spontaneous sensitization to CJ pollen than Cry j 2 in dogs. It was also found that in atopic dogs sensitive to CJ pollen, clinical aggravation was associated with an increase of the serum IgE level directed to Cry j 1 during the season of CJ pollination (Masuda et al., 2002), emphasizing the importance of Cry j 1 as a possible cause of atopic dermatitis in dogs in Japan. Therefore, it can be considered that Cry j 1 should be a target for development of immunotherapy.

In this study, in order to identify T-cell epitopes of Cry j 1 in dogs, lymphocyte blastogenic responses to overlapping peptides of Cry j 1 were examined in dogs both experimentally or spontaneously sensitized to CJ pollen.

2. Materials and methods

2.1. Dogs experimentally sensitized to Japanese cedar pollen allergen

For experimental sensitization with CJ pollen antigen, 22 healthy dogs kept for experimental purposes were used. All the dogs were female beagles whose age ranged from 6 months to 1 year. The dogs were experimentally sensitized to the antigen of CJ pollen by subcutaneous injection of 100 µg of crude CJ pollen extract in conjunction with 20 mg of alum as reported previously (Yamashita et al., 2000). After injections of the alum-mixed CJ pollen extract twice with a 2-week interval, intradermal testing and IgE testing for crude antigen of CJ pollen and Cry j 1 were carried out in order to examine the successful sensitization with CJ pollen. All the dogs showed positive results in the both testings (Table 1). As a control group, three healthy female beagles with age of 8–10 months were also used. The control dogs were negative for CJ pollen antigen and Cry j 1 in the intradermal testing.

Table 1

Results of antigen-specific IgE testing, intradermal testing, and lymphocyte blastogenic responses to CJ pollen extract and Cry j 1 in dogs experimentally sensitized to CJ pollen

Dog no.	Intradermal testing		IgE testing (U/ml)		Lymphocytes
	CJ pollen	Cry j 1	CJ pollen	Cry j 1	CJ pollen
E1	3+	3+	4250	2380	5.4
E2	3+	3+	28600	4770	4.4
E3	3+	3+	3330	565	4.9
E4	3+	3+	303	59	2.8
E5	3+	3+	739	314	6.8
E6	3+	3+	1090	812	3.9
E7	3+	3+	1910	949	3.1
E8	3+	3+	1420	17700	2.4
E9	3+	3+	14900	11500	2.3
E10	3+	3+	17200	5610	4.3
E11	3+	3+	18100	11600	3.7
E12	3+	3+	6240	5760	3.8
E13	3+	3+	423	139	2.2
E14	3+	3+	12000	4370	3.5
E15	3+	3+	36200	12400	2.4
E16	3+	3+	18500	7810	2.9
E17	3+	3+	16200	13000	14.5
E18	3+	3+	3270	2870	15.2
E19	3+	3+	10200	3100	3.8
E20	3+	3+	7800	6820	3.3
E21	3+	3+	8050	6070	3.5
E22	3+	3+	20400	6570	19.6

2.2. Atopic dogs spontaneously sensitive to Japanese cedar pollen allergen

Five dogs with pruritic skin disease and/or external otitis were diagnosed with atopic dermatitis according to Willemse's criteria (Willemse, 1986) for the clinical diagnosis of atopic dermatitis in dogs, as shown previously (Masuda et al., 2000a,c, 2002). Other skin diseases caused by parasite infestation (*Demodex canis*, scabies, and fleas), bacterial or fungal infection, or dermatophytosis were excluded based on the results of routine dermatological laboratory tests including cytological examination of surface samples, skin scrapings and trichography. Food hypersensitivity was excluded based on the unresponsiveness to a restricted diet and lack of clinical history associated with a particular food intake. The dogs were examined by intradermal testing and IgE testing as described below and found to be sensitive only to CJ pollen allergen, but not to other allergens (Table 2).

2.3. Intradermal testing

Intradermal testing was carried out as we reported previously (Masuda et al., 2000a). For screening of positive allergens in atopic dogs, 24 extracts of common allergens not including crude CJ pollen extract and Cry j 1 were purchased from a commercial company (Greer Laboratories, Lenoir, NC, USA). Crude CJ pollen extract and Cry j 1 were prepared as reported previously (Yasueda et al., 1983) and used for intradermal testing at a concentration of 200 ng/ml (Masuda et al., 2000c, 2002). The diameters of reactive wheals were measured 15–20 min after intradermal injection. The test sites were graded as follows: 3+, equal to or greater than the diameter of the positive control; 2+, equal to or greater than the mean diameter of the positive and negative controls; 1+, larger than the diameter of the negative control but smaller than the mean diameter of the positive and negative controls; –, equal to or smaller than the diameter of the negative control. Intradermal reactions

Table 2

Atopic dogs naturally sensitive to CJ pollen

Dog no.	Breeds	Sex	Age (years)	Intradermal testing	IgE (Immunodot)
S1	Yorkshire Terrier	SF	3	3+	+
S2	West Highland White Terrier	SF	6	3+	+
S3	Cavalier King Charles Spaniel	SF	6	3+	+
S4	Shi Zhu	CM	10	3+	+
S5	Labrador Retriever	SF	3	3+	+

All the dogs showed positive reactions to crude CJ pollen extract in intradermal testing and IgE testing (Immunodot). SF, spayed female; CM, castrated male.

graded as 2+ or 3+ were considered to be positive reactions.

2.4. IgE testing

As a screening system for serum antigen-specific IgE in the atopic dogs, two commercially available test kits (Topscreen and Immunodot, CMG Laboratories, Fribourg, Switzerland) were used to measure IgE against 28 kinds of allergens as previously described (Derer et al., 1998; Masuda et al., 2000a). For the quantitative measurement of IgE, the concentrations of IgE specific for crude CJ pollen antigen and Cry j 1 were also assessed by a fluorometric ELISA as described previously (Masuda et al., 2000c, 2002).

2.5. Overlapping peptides of Cry j 1

The series of 35 overlapping peptides of Cry j 1 used in this study were synthesized with a solid-phase peptide synthesizer (Protein Technologies Inc., Tucson, AZ, USA) to cover the entire amino acid sequence of Cry j 1 (Fig. 1) (Tamura et al., 2000). Each peptide consisted of 20 amino acids corresponding to a portion of the amino acid sequence of Cry j 1. The peptides were purified by HPLC and the amino acid sequences were confirmed using a peptide sequencer (Hirahara et al., 1998, 2001). Each peptide was used to test the lymphocyte blastogenic response at the concentration of 1 µg/ml in culture medium.

2.6. Lymphocyte blastogenic responses

Tests for lymphocyte blastogenic responses were carried out as we previously reported (Masuda et al., 2000b, 2002). Briefly, peripheral blood mononuclear cells (PBMCs) from dogs were cultured in the pre-

sence of 5 µg of crude antigen of CJ pollen and 1 µg of Cry j 1 or overlapping peptide per ml in a humidified atmosphere of 5% CO₂ at 37 °C for 3 days. ³H-thymidine-incorporation during the final 18 h of the culture was measured using a scintillation counter. A stimulation index equal to or greater than 2.0 was judged to be positive for the lymphocyte blastogenic response reaction (Masuda et al., 2000b, 2002).

3. Results

3.1. Experimental sensitization with CJ pollen allergen in dogs

Experimental sensitization to CJ pollen allergen was shown to be successfully induced in the 22 dogs (E1–E22) that had been injected with crude CJ pollen extract and alum, based on positive reactions to crude CJ pollen extract and Cry j 1 in intradermal testing and IgE testing. In accordance to the strong positive results in intradermal testing, high levels of IgE against crude CJ pollen extract (303–36 200 U/ml, mean ± standard deviation: 10 506 ± 9820 U/ml) and Cry j 1 (59–17 700 U/ml, 5690 ± 4930 U/ml) were detected by fluorometric ELISA in the 22 dogs. The lymphocyte blastogenic responses against CJ pollen extract were also positive in all the dogs (Table 1), which made it possible to perform the further experiments for identification of T-cell epitopes of Cry j 1 using the dogs. The stimulation indices of lymphocyte blastogenic responses to crude CJ pollen extract ranged from 2.2 to 19.6 (5.4 ± 4.70), indicating that the procedures of the lymphocyte blastogenic responses were successfully completed.

In three control dogs, no positive lymphocyte blastogenic responses directed to crude antigen of CJ

No. of AAs	Peptide No.	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E13	E14	E15	E16	E17	E18	E19	E20	E21	E22	positive cases	positive %
1-20	1																							0	0
11-30	2																							1	4.5
21-40	3																							1	4.5
31-50	4																							0	0
41-60	5																							1	4.5
51-70	6																							2	9.1
61-80	7																							3	13.6
71-90	8																							9	40.9
81-100	9																							0	0
91-110	10																							11	50
101-120	11																							9	40.9
111-130	12																							0	0
121-140	13																							0	0
131-150	14																							0	0
141-160	15																							0	0
151-170	16																							0	0
161-180	17																							3	13.6
171-190	18																							0	0
181-200	19																							2	9.1
191-210	20																							0	0
201-220	21																							0	0
211-230	22																							1	4.5
221-240	23																							0	0
231-250	24																							0	0
241-260	25																							0	0
251-270	26																							2	9.1
261-280	27																							0	0
271-290	28																							0	0
281-300	29																							0	0
291-310	30																							3	13.6
301-320	31																							1	4.5
311-330	32																							0	0
321-340	33																							0	0
331-350	34																							1	4.5
341-353	35																							0	0
Cry j 1																								22	

Fig. 1. Lymphocyte blastogenic response to 35 kinds of overlapping peptides of Cry j 1 and whole Cry j 1 extract in 22 dogs experimentally sensitized to CJ pollen antigen. Black boxes indicate positive lymphocyte blastogenic responses with showing stimulation indices equal to or greater than 3.0. Grey boxes show positive lymphocyte blastogenic responses with stimulation indices of equal to or greater than 2.0 and less than 3.0. White boxes show negative lymphocyte blastogenic responses with stimulation indices less than 2.0.

pollen, Cry j 1, and any of the overlapping peptides were detected. The stimulation indices were 1.15–1.48, 0.97–1.66, and 0.34–1.57 after stimulation with crude CJ pollen extract, Cry j 1, and the series of overlapping Cry j 1 peptides, respectively.

3.2. Lymphocyte blastogenic response to overlapping peptides of Cry j 1 in dogs experimentally sensitized with CJ pollen extract

In the 22 dogs experimentally sensitized with CJ pollen extract, lymphocyte blastogenic responses were detected in the presence of Cry j 1-overlapping peptides, Nos. 2, 3, 5–8, 10, 11, 17, 19, 22, 26, 30, 31, and 34, with stimulation indices ranging from 2.0 to 27.6 (Fig. 1). Most of the positive reactions were found in the responses against N-terminal peptides (Nos. 2–11, p11–120) of Cry j 1. Among the peptides, those that

frequently induced apparent lymphocyte blastogenesis in a high proportion of the dogs were Nos. 8 (p71–90) (9 dogs, 41%), 10 (p91–110) (11 dogs, 50%), and 11 (p101–120) (9 dogs, 41%), indicating that these peptides contained major T-cell epitopes of Cry j 1 in the dogs experimentally sensitized with CJ pollen extract. For the other peptides that induced positive responses of lymphocyte blastogenesis, the number of the dogs that showed apparent blastogenesis was 3 or less, indicating that these peptides contained minor T-cell epitopes of Cry j 1 in these dogs.

3.3. Lymphocyte blastogenic responses to overlapping peptides of Cry j 1 in atopic dogs spontaneously sensitive to CJ pollen

The breeds of the atopic dogs spontaneously sensitive to CJ pollen varied, and four of the five dogs were

spayed females while one dog was a castrated male (Table 2). The age of the dogs ranged from 3 to 10 years.

All of the five atopic dogs showed lymphocyte blastogenic responses in the presence of Cry j 1 with stimulation indices (SI) ranging between 2.0 and 13.9, indicating that the lymphocyte blastogenic responses were successfully measured in this study. Overlapping peptides such as Nos. 1–3, 7, 10, 13, 14, 16, 21, 25, 26, 27, 31, 32, and 34 were shown to induce a lymphocyte blastogenic response (SI, 2.1–18.8) in one or two of the five dogs spontaneously sensitive to CJ pollen (Fig. 2). None of the peptides induced a lymphocyte

blastogenic response with SI equal to or greater than 2.0 in more than two of the five dogs examined.

4. Discussion

In human patients with CJ pollinosis, 3 major T-cell epitopes of Cry j 1 have been identified and shown to be distributed over the whole sequence of Cry j 1 (Sone et al., 1998). As indicated in this study, T-cell epitopes of Cry j 1 tended to be located in the N-terminal one-third of amino acid sequence of Cry j 1 (Nos. 2–11) in dogs experimentally sensitized to CJ pollen extract. Interestingly, the peptides (Nos. 8, 10 and 11) induced lymphocyte blastogenesis in 41–50% of the dogs examined in this study, suggesting that these peptides contained major T-cell epitopes of Cry j 1 in these particular dogs. The fact that the peptide No. 11 was also reported to contain a dominant T-cell epitope in humans with CJ pollinosis (Sone et al., 1998) suggests similarity between dogs and humans with respect to the antigen recognition of T cells.

It was found that the peptides inducing positive lymphocyte blastogenic responses in this study were not completely consistent among the atopic dogs spontaneously sensitive to CJ pollen antigen, suggesting that there was a wide variation of T-cell epitopes of Cry j 1 in the dogs. Although the number of dogs examined in this study was limited, this variation of the epitopes may be due to a difference in dog breeds, since several T-cell epitopes could be identical among the beagles that used for experimental sensitization to CJ pollen allergen in this study. The variation of T-cell epitopes might be caused by variation of the type of MHC class II molecules. T-cell epitopes of Cry j 1 and Cry j 2 in human patients with CJ pollinosis are known to be restricted by MHC class II molecules (Sone et al., 1998, 1999). In Japanese people with CJ pollinosis, there was an association between the type of HLA class II molecule and sensitization to CJ pollen allergen (Hori et al., 1996; Matsushita et al., 1987). These data suggested that certain types of MHC class II molecule would be more prone to recognize CJ pollen allergen. Although MHC class II molecules have not been well characterized in dogs, it will be worth investigating the genetic background of the dogs with spontaneous CJ pollinosis in terms of the association

No. of AAs	Peptide No.	S1	S2	S3	S4	S5
1- 20	1					
11- 30	2					
21- 40	3					
31- 50	4					
41- 60	5					
51- 70	6					
61- 80	7					
71- 90	8					
81- 100	9					
91- 110	10					
101- 120	11					
111- 130	12					
121- 140	13					
131- 150	14					
141- 160	15					
151- 170	16					
161- 180	17					
171- 190	18					
181- 200	19					
191- 210	20					
201- 220	21					
211- 230	22					
221- 240	23					
231- 250	24					
241- 260	25					
251- 270	26					
261- 280	27					
271- 290	28					
281- 300	29					
291- 310	30					
301- 320	31					
311- 330	32					
321- 340	33					
331- 350	34					
341- 353	35					
	Cry j 1					

Fig. 2. Lymphocyte blastogenic response to 35 kinds of overlapping peptides of Cry j 1 and whole Cry j 1 extract in five atopic dogs spontaneously sensitive to CJ pollen. Black boxes indicate positive lymphocyte blastogenic responses with stimulation indices equal to or greater than 3.0. Grey boxes show positive lymphocyte blastogenic responses with stimulation indices equal to or greater than 2.0 and less than 3.0. White boxes show negative lymphocyte blastogenic responses with stimulation indices less than 2.0.

between the type of MHC class II molecule and T-cell epitopes of CJ pollen allergen.

Specific immunotherapy using whole allergen extracts has been applied to humans with allergic diseases for many years, but its adverse effects, especially anaphylaxis against the injected allergen extracts, have been problematic during the treatment (Muller et al., 1992). Recently, immunotherapy with peptides containing major T-cell epitopes and lacking sites recognized by IgE has provided an alternative strategy to decrease side effects and to induce T-cell tolerance in an antigen-specific manner (Hirahara et al., 2001; Marcotte et al., 1998; Norman, 1993). In a mouse model, it has been shown that the immune response to allergens can be controlled by a peptide therapy, resulting in a shift of the allergic reaction to Th1-skewed immune response (Astori et al., 2000) and prevention of anaphylaxis (von Garnier et al., 2000). Unfortunately, in this study, Cry j 1 T-cell epitopes were not completely identical in the beagle. In atopic dogs, five kinds of different dog breeds were examined, showing that Cry j 1 T-cell epitopes were distributed over the amino acid sequence of Cry j 1. Thus, T-cell epitopes consistent in the dogs were not identified in this study; however, it could be proposed that a peptide immunotherapy using T-cell epitopes in each dog would be plausible as a tailor-made treatment.

The dogs experimentally sensitized to Japanese cedar pollen allergen did not show atopic dermatitis as a clinical sign of allergic reaction to the pollen. A previous study showed that the experimentally sensitized dogs had Th2-skewed cytokine profile of lymphocytes reactive to the pollen allergen, indicating the successful establishment of allergic reactions to the pollen allergen in the dogs (Fujiwara et al., 2003). We also found that these dogs showed bronchial hyper-reactivity to the pollen exposure (unpublished data). Th1/Th2 cytokine profile in the skin of atopic dogs was still controversial (Maeda et al., 2002; Nuttall et al., 2002; Olivry et al., 1999). Therefore, it is conceivable that manifestation of atopic dermatitis may require other factor(s) than Th2-type reaction specific to sensitizing allergen(s).

In conclusion, T-cell epitopes of Cry j 1 in dogs were identified in this study. In the experimental model in which dogs were sensitized to CJ pollen, three peptides in Cry j 1 were identified as major T-cell

epitopes; however, in the five atopic dogs naturally sensitive to CJ pollen allergen, there was no consistent peptide that induced positive lymphocyte blastogenesis. The results in this study will provide useful information to develop a peptide therapy for the dogs sensitive to CJ pollen allergen.

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