

Immunological Findings in 3 Dogs Clinically Diagnosed with Allergic Rhinitis

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ABSTRACT. Three dogs clinically diagnosed with allergic rhinitis (AR) were examined for their immunological findings. House dust mites (HDM) such as *Dermatophagoides farinae* (DF) and *D. pteronyssinus* (DP) were identified as positive allergens in the 3 dogs with both intradermal skin test and serum antigen-specific IgE test. Lymphocyte blastogenic response of peripheral blood mononuclear cells (PBMCs) under stimulation with DF antigen in dogs with AR was higher than that in 4 healthy control dogs. Expression level of IL-4 mRNA in PBMCs obtained from the 3 AR dogs was higher than that in PBMCs obtained from 4 healthy control dogs before and after stimulation with DF antigen. Expression level of IFN- γ mRNA in PBMCs was not different between the AR and control dogs before and after stimulation with DF antigen. These results suggested that allergic reaction to HDM antigen and T_H2-type immune response were associated with the development of AR in 3 dogs examined in this study.

KEY WORDS: allergic rhinitis, canine, house dust mite, T_H2-type immune response.

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Allergic rhinitis (AR) is known to be one of the common allergic diseases in humans. Human AR has been characterized by its respiratory symptoms such as sneezing, rhinorrhea and nasal congestion that are provoked by IgE-mediated inflammation [11]. Atopy is known as an important factor for the development of AR in humans [17,18]. Among the environmental allergens, house dust mite (HDM), plant pollens, and fungi have been identified as common allergens in humans with AR in Japan [13]. Production of IL-4 by allergen-stimulated T cells was shown to be higher in patients with AR than unaffected people [2], suggesting that T_H2 immune response to the environmental allergens plays an important role in the development of AR in humans.

Although dog cases suspected to have AR are sometimes observed in small animal practice, immunological characteristics of canine AR is not well understood. MacDougall reported that a dog with upper respiratory symptoms showed positive reactions to antigens of house dust, grasses and trees in intradermal skin test (IDST), and the symptoms were improved by immunotherapy with alum-precipitated extracts of these antigens [9]. On the other hand, allergic reactions in canine atopic dermatitis (AD) have been more extensively investigated. Environmental allergens have been identified with IDST and IgE test in dogs with AD. In Japan, HDM was identified as the most common allergens in canine AD [6]. Peripheral blood mononuclear cells (PBMCs) obtained from dogs with AD were shown to proliferate under stimulation with the extracts of allergens [12]. These data suggest that allergic reactions directed to envi-

ronmental allergens can occur in dogs and are associated with various allergic diseases in small animal practice.

In this study, in order to investigate an association of canine AR with allergic reactions, we carried out examinations of positive allergens with IDST and serum IgE test in 3 dogs clinically diagnosed with AR. Furthermore, we carried out experiments on the lymphocyte blastogenic responses and cytokine profiles in PBMCs obtained from the 3 dogs.

MATERIALS AND METHODS

Animals: Three dogs (Dogs 1, 2, and 3) were referred to the Veterinary Medical Center of the University of Tokyo because of perennial sneezing and rhinorrhea (Table 1). All of the 3 dogs had received combined vaccines for infections of virus such as canine distemper virus, parvovirus, and adenovirus every year. All of the 3 dogs had showed the nasal symptoms for more than 1 year. The clinical symptoms in the 3 dogs did not improve after treatment with antibiotics such as cefalexin, enrofloxacin and clarithromycin at optimal dosages for 4 weeks, however, quickly diminished by oral administration of prednisolone at a dosage of 1 mg/kg, q24 hr or anti-histaminics, clemastine fumarate at a dosage of 0.1–0.2 mg/kg, q12 hr. Relapses of the nasal symptoms were observed after discontinuance of the corticosteroids or anti-histaminics in all the 3 dogs. No dermatological symptom was observed in these 3 dogs. Other diseases including infections with microorganisms (bacterial, viral and mycotic infections), nasal mite infestation, trauma, foreign body, dental disease, nasal neoplasm, and congenital abnormality were excluded based on the clinical histories and findings in various examinations such as complete blood cell count, serum biochemistry, cytology and fungal culture of the

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Table 1. Three dogs diagnosed with allergic rhinitis examined in this study

Dog No.	Breed	Sex	Age (yrs)	Increased eosinophils in nasal discharge	Positive allergens	
					IDST ^{a)}	IgE test
1	Golden retriever	NM ^{b)}	3	Negative	DF ^{d)} , DP ^{e)}	HDM ^{f)} , Storage mites
2	Shetland sheep dog	NM ^{b)}	9	Negative	DF ^{d)} , DP ^{e)}	HDM ^{f)}
3	Miniature dachshund	F ^{c)}	10	Negative	DF ^{d)} , DP ^{e)}	HDM ^{f)}

a) IDST, intradermal skin test, b) NM, Neutered male, c) F, female, d) DF, *Dermatophagoides farinae*, e) DP, *Dermatophagoides pteronyssinus*, f) HDM, House dust mites.

nasal discharge, nasal radiography, rhinoscopy, and computed tomography (CT) of the nasal cavities. Based on the clinical symptoms, responses to glucocorticoid treatment, and exclusion of other diseases from the results of the various examinations, the 3 dogs were clinically diagnosed with allergic rhinitis.

Four healthy 1-year-old females beagles kept in a clean room for experimental purposes were used as a control group for the examinations of blastogenic response and cytokine mRNA expression in their PBMCs. These control dogs did not show any clinical sign.

Intradermal skin test: In intradermal skin test (IDST), 26 kinds of allergen extracts were used as previously reported [6]. The allergen extracts used in this study were purchased from a commercial company (Greer Laboratory, Lenoir, NC) except for Japanese cedar pollen (*Cryptomeria japonica*, CJ) antigen, which was prepared as reported previously [19]. Briefly, 8 groups of allergens were used: HDM (*Dermatophagoides farinae*: DF and *Dermatophagoides pteronyssinus*: DP), arthropods (flea, insect mix), cat epithelia, foods (corn, rice, wheat, beef, milk, egg, etc), mold (*Aspergillus* mix, *Penicillium* mix, etc), grasses (Yellow dock, Sage mix, and 7 grass mix), weeds (National weed mix), and trees (Elm mix, Pine mix, Eastern oak mix, and Birch mix). The allergen extracts except HDM, flea and CJ antigens were diluted to 1000PNU/ml with a diluent containing 0.9% sodium chloride and 0.4% phenol and injected into the skin of the ventral thorax at the dosage of 0.05 ml with a 26-gauge syringe under sedation. HDM and flea antigens were diluted to 1:5000 w/v and CJ antigen was diluted to 200 ng/ml to use for IDST. The diluent also was used as a negative control. Histamine phosphate (0.0275 mg/ml) was used as a positive control. The diameter of wheal was measured 15 min after injection and a wheal equal to or greater than the diameter of the positive control was interpreted as a positive reaction.

Serum antigen-specific IgE test: Serum antigen-specific IgE test in dogs was performed using two commercially available test kits, Topscreen and Immnodot (CMG/HESKA Allergy Products, Fribourg, Switzerland) as previously reported [20]. In this test, allergens or allergen mixtures were located on nitrocellulose strips. Each strip had dog-IgE as a positive control and also had a negative control. In this assay system, mixtures of allergens were used for the screening assay of five allergen groups, including indoor (house dust mites: *D. farinae* and *D. pteronyssinus*, storage mites, flea, human dander, and cat dander), outdoor (six

grasses mix, tree mix; birch, oak and hazel, mugwort / plantain, olive / parietaria, and Japanese cedar), foods (cow milk, egg, soybean, maize, wheat, beef, sheep, pork, fish mix, and peanut), molds (*Alternaria tenuis*, *Aspergillus fumigatus*, *Cladosporium herbarum*, *Penicillium notatum*, and *Candida albicans*), while the Immunodot test was used to detect IgE specific to 24 individual antigens in each allergen group. Allergens showing positive reactions were recorded in Table 1.

Lymphocyte blastogenic response: Lymphocyte blastogenic response was measured using PBMCs from the 3 dogs with AR and 4 control dogs as previously described [7]. House dust mites such as *Dermatophagoides farinae* (DF) and *D. pteronyssinus* (DP) were shown to be positive in both IDST and IgE test in all the 3 dogs with AR and were therefore used as stimulators for measurement of blastogenic response. PBMCs were cultured with the antigens at a concentration of 1:10,000 w/v for 96 hr at 37°C. Concanavalin A (Con A) was used at a concentration of 5 µg/ml as a positive control and an unstimulated group was prepared as a negative control. Incorporation of ³H-thymidine (37 kBq/ml) for the final 18 hr was measured using a gamma scintillation counter. Stimulation indices (SI), ratios of mean cpm in the antigen-stimulated group to mean cpm in the negative control group, were calculated.

Quantitative real-time sequence detection system to measure mRNA expression: Total RNA samples were extracted from freshly isolated PBMCs and PBMCs that had been cultivated in the presence or absence of DF antigen (Greer Laboratory) at a concentration of 1:10,000 w/v at 37°C for 24 hr with the acid guanidium-phenol-chloroform (AGPC) method with RNazol (Tel Test, Friendswood, TX). The extracted RNA samples were treated with RNase-free DNase I (Invitrogen, Carlsbad, CA) to remove DNA contamination and then stored at -80°C until use.

A real-time sequence detection system was applied for quantitative measurement of cytokine mRNAs as previously reported [4]. The cytokines examined in this study were IL-4 and IFN-γ and the mRNA amounts of these cytokines were quantified by comparing with that of β-actin in each sample. For each of the targeted genes (canine IL-4, IFN-γ and β-actin), a pair of oligonucleotide primers and an oligonucleotide probe were designed based on the sequences in GenBank/EMBL/DDBJ database (GenBank/EMBL/DDBJ accession numbers: IL-4, AF054833; IFN-γ, AF126247; β-actin, Z70044) using a computer software package, Primer Express software (Applied Biosystems, Foster City, CA)

Table 2. Sequences of primer pairs and probes for quantitative real-time sequence detection system in this study

Target gene	Primer Probe	Sequence (5'-3')	Nucleotide position	Length of the PCR product (bp)	GeneBank accession number
IL-4	Forward primer	CATCCTCACAGCGAGAACG	117-136	83	AF054833
	Reverse primer	CCTTATCGCTTGTGTTCTTTGGA	177-199		
	Probe	CATGGAGCTGACTGTCAAGGACGTCCTCA	144-172		
IFN- γ	Forward primer	GCGCAAGGCGATAAATGAAC	336-355	82	AF126247
	Reverse primer	CTGACTCCTTTTCCGCTTCCT	397-417		
	Probe	TGATGAATGATCTCTACCAAGATCCAACC	365-394		
β -actin	Forward primer	GACCCTGAAGTACCCCATTTGAG	131-152	81	Z70044
	Reverse primer	TTGTAGAAGGTGTGGTGCCAGAT	189-211		
	Probe	CGTCACCAACTGGGACGACATGGA	161-184		

(Table 2). The PCR products amplified with each primer pair were sequenced to confirm the amplification of each targeted cytokine cDNA. A CT (threshold cycle) value was defined as the PCR cycle at which a significant increase in reporter fluorescence was first detected, and used for quantification of mRNA expression of cytokines. The CT value of the calibrator (β -actin) was subtracted from that of the target cytokine to calculate the CT value. All samples were examined in duplicate and the mean value of the CT was calculated in each sample. The amount of the targeted RNAs was represented as the $2^{-\Delta CT}$ value, resulting in evaluation of the samples as an n-fold difference relative to β -actin.

All of the immunological test except IgE test in this study were carried out at least 1 week after discontinuance of the administration of the corticosteroids and anti-histaminics.

RESULTS

IDST: In IDST, all the 3 dogs showed positive reactions to antigens of DF and DP, but were negative to all of other 24 antigens (Table 1). Four healthy control dogs kept for experimental purposes in a clean room were negative to all of the 26 antigens.

Serum antigen-specific IgE test: In serum antigen-specific IgE test, all of the 3 dogs showed positive reactions to indoor antigens in the screening test (Topscreen) and were shown to be positive to HDM in the antigen-specific test (Immunodot) (Table 1). In Dog 1, serum IgE directed to storage mites was also detected. Serum samples obtained from the 4 healthy control dogs were negative to all of the antigen groups in the screening kit, topscreen.

Lymphocyte blastogenic response: In lymphocyte blastogenic response, mean $\pm$ standard error of mean (SEM) values of SI after stimulation with antigens of DF and DP in the 3 dogs with AR were 5.2 ± 2.2 (Dog 1, 9.6; Dog 2, 2.3; Dog 3, 3.8) and 2.8 ± 1.3 (Dog 1, 3.4; Dog 2, 0.3; Dog 3, 4.7), respectively. Mean $\pm$ SEM values of SI after stimulation with antigens of DF and DP in control dogs were 1.2 ± 0.3 and 1.0 ± 0.5 , respectively (Fig. 1). Mean $\pm$ SEM values of SI after cultivation with Con A were 96.4 ± 29.9 in the 3 dogs with AR, and 124.0 ± 71.9 in the control dogs.

Expression of mRNAs of IL-4 and IFN- γ in freshly iso-

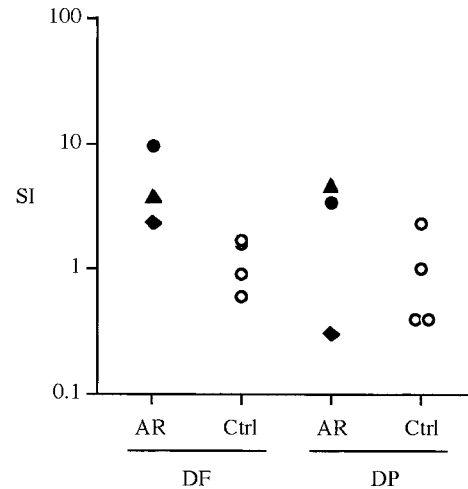


Fig. 1. Lymphocyte blastogenic responses to antigens of DF and DP in 3 dogs with allergic rhinitis (AR) (Dog 1, ●; Dog 2, ◆; Dog 3, ▲) and 4 control dogs (Ctrl). Stimulation indices (SI), ratios of mean cpm in the antigen-stimulated group to mean cpm in the negative control group, were calculated.

lated PBMCs: Expression of IL-4 mRNA was detected in freshly isolated PBMCs from all the 3 dogs with AR as shown by the value of $2^{-\Delta CT}$ in comparison to the amount of β -actin mRNA (mean $\pm$ SEM, $2.3 \times 10^{-6} \pm 1.2 \times 10^{-6}$) (Fig 2A). In contrast, the amount of IL-4 mRNA in freshly isolated PBMCs from control dogs was below the lower detection limit of this assay. IFN- γ mRNA in freshly isolated PBMCs was detected in both of the AR and control dogs and the levels were similar between the 2 groups (AR dogs, $3.3 \times 10^{-3} \pm 1.6 \times 10^{-3}$; control dogs, $1.0 \times 10^{-3} \pm 4.6 \times 10^{-4}$) (Fig 2A).

Expression of mRNAs of IL-4 and IFN- γ in PBMCs after antigen-stimulation: Since lymphocyte blastogenic responses to DF antigen tended to be high in the dogs with AR, quantitative analysis of cytokine mRNA in PBMCs was also performed after stimulation with DF antigen. The expression level of IL-4 mRNA in the PBMCs from dogs with AR after stimulation with DF antigen ($3.4 \times 10^{-5} \pm 2.6$

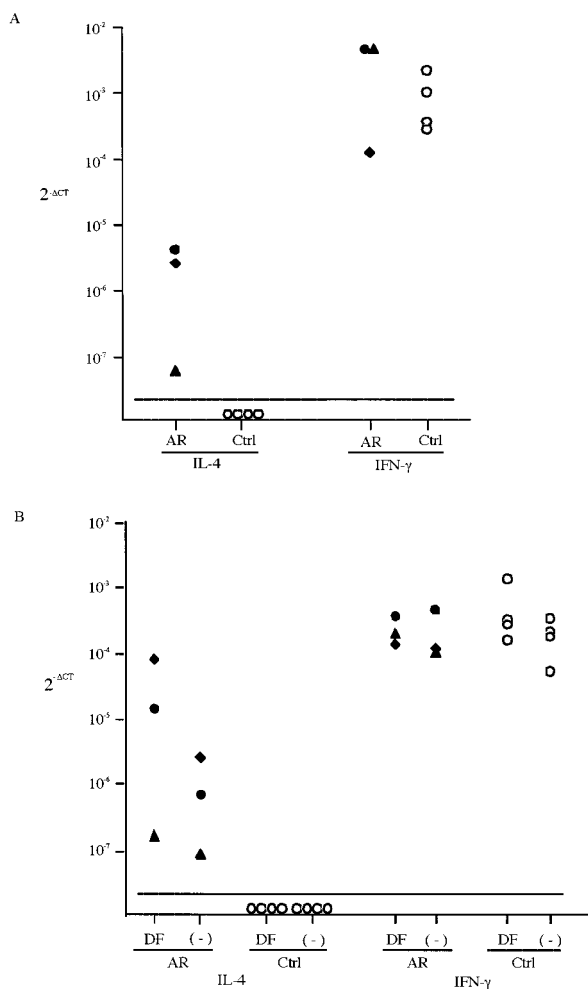


Fig. 2. (A) Expressions of IL-4 and IFN- γ mRNAs in freshly isolated PBMCs from 3 dogs with AR (Dog 1, ●; Dog 2, ◆; Dog 3, ▲) and 4 control dogs (○). (B) Expressions of IL-4 and IFN- γ mRNAs in PBMCs stimulated with DF antigens (1:10,000 w/v) and unstimulated PBMCs from 3 dogs with AR (Dog 1, ●; Dog 2, ◆; Dog 3, ▲) and 4 control dogs (○). The horizontal lines indicate the lower detection limit of the assay.

$\times 10^{-5}$) was shown to be higher than that without stimulation with DF antigen ($1.2 \times 10^{-6} \pm 7.1 \times 10^{-7}$) (Fig. 2B), whereas the expression level of IL-4 mRNA in PBMCs from control dogs was below the lower detection limit in both of the DF antigen-stimulated and unstimulated samples.

The IFN- γ mRNA levels in the PBMCs cultured without DF antigen were similar between the AR group ($2.4 \times 10^{-4} \pm 1.3 \times 10^{-4}$) and control group ($2.0 \times 10^{-4} \pm 6.1 \times 10^{-5}$) (Fig. 2B). After stimulation with DF antigen, the expression level of IFN- γ mRNA in the PBMCs from dogs with AR ($2.4 \times 10^{-4} \pm 7.0 \times 10^{-5}$) tended to be lower than that from control dogs ($5.7 \times 10^{-4} \pm 3.0 \times 10^{-4}$) (Fig. 2B).

DISCUSSION

In the present study, it was demonstrated that the 3 dogs clinically diagnosed with allergic rhinitis were sensitive to DF and DP antigens by both IDST and serum antigen-specific IgE test. Analysis of the lymphocyte blastogenic response in this study indicated that lymphocytes reactive to HDM antigens were circulating in the peripheral blood of the 3 dogs diagnosed with AR, which also supported the fact that the dogs with AR were sensitized with the HDM antigens. HDM antigens have been identified as common allergens in allergic diseases in humans [3, 13, 16] and canine atopic dermatitis [1, 6, 10, 15, 21]. Since HDM usually exist in indoor environments throughout a year [8], the clinical symptoms of HDM allergy should be seen in all season. Since the symptoms of the 3 dogs diagnosed with AR examined in this study occurred perennially, being consistent with the positive results in tests for the allergic reactions to HDM antigens.

Profiles of cytokine mRNAs in this study revealed the IL-4 mRNA expression in freshly isolated PBMCs from the 3 dogs diagnosed with AR. The expression level of IL-4 mRNA increased after DF antigen stimulation in the dogs with AR, whereas expression of IL-4 mRNA in the control dogs was undetectable in this assay even after the DF antigen stimulation. These findings suggested that allergen-reactive T cells circulating in the peripheral blood in the 3 dogs diagnosed with AR might be polarized to be T_H2 -type in terms of the cytokine production. These findings were similar to those in human AR, since IL-4 production in PBMCs from AR patients was found to increase after allergen stimulation [2]. The expression levels of IFN- γ mRNA did not significantly differ between the AR dogs and control dogs in this study. From these results, it was likely that the cytokine profile skewed to T_H2 -dominant (IL-4 dominant) type was associated with the pathogenesis of canine AR.

An appropriate animal model of AR will help to develop novel therapies for human AR. There have been a limited number of reports on spontaneous allergic rhinitis in animals. Although reports on spontaneous rhinitis associated with CJ pollinosis in the monkey [14] and cat [5] revealed *in vivo* and *in vitro* evidences of type I hypersensitivity to CJ pollen antigen in the affected animals, there has been no report on spontaneous allergic rhinitis attributed to HDM in animals. In the dog, as shown in the present study, AR might be provoked by sensitization to HDM with cytokine production skewed to T_H2 -type. Since all the AR dogs examined in this study were kept indoors by their owners, the condition of sensitization to HDM in the dogs would reflect environments in which the sensitization to HDM could occur in humans. In this sense, dogs with spontaneous AR can be recognized as an appropriate animal model for human AR. Further studies such as nasal provocation test and rhinomanometry should be carried out to provide direct *in vivo* evidence indicating an association between the allergic reaction to HDM and development of clinical symptoms in canine AR.

In conclusion, positive reactions to HDM antigens were detected in 3 dogs clinically diagnosed with allergic rhinitis by IDST, IgE test, and lymphocyte blastogenic response. In addition, the expression level of IL-4 mRNA in PBMCs was higher in the 3 dogs diagnosed with AR than control dogs, and it was also enhanced by stimulation with DF antigen. These results suggested that the allergic reaction to HDM antigens could be associated with the development of AR in dogs.

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