Determination of Antigenic Proteins of Housedust Mites in 90 Dogs Suffering from Atopic Dermatitis

Kohei YAMASHITA¹⁾, Chiharu FUJIWARA¹⁾, Ryouji AZUMA²⁾, Takeshi SAWAZAKI¹⁾, Yoshiki NAKAO¹⁾ and Atsuhiko HASEGAWA³⁾

¹⁾Pharmaceutical Research Laboratory, Hitachi Chemical Co., Ltd., 4-13-1 Higashi-cho, Hitachi-shi, Ibaraki 317-8555, ²⁾Azuma Animal Hospital, Tokyo 202-0011 and ³⁾Department of Pathobiology, Nihon University School of Veterinary Medicine, Kanagawa 252-0813, Japan

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ABSTRACT. Housedust mites, *Dermatophagoides pteronyssinus* (*D. pteronyssinus*) and *Dermatophagoides farinae* (*D. farinae*), are the important causative agents of allergic diseases in human and animals. By using 165 dogs suffering from atopic dermatitis (AD), serum levels of immunoglobulin E (IgE) antibody against 25 kinds of allergen including housedust mites were determined. Housedust mites were the most frequent allergen against which 90 of the 165 allergic dogs (54.5%) by IMMUNODOT assay. With the further analysis of immunoblotting assay in the 90 dogs sensitized with housedust mites, antigenic proteins of housedust mites recognized by IgE antibodies were with the apparent molecular masses of 15, 76, 90, 98, and 170-kD. Among them, the 15-kD protein that might be identical to Group 2 antigens (Der f2, Der p2) was prominently observed (52/90). This study indicates that about a half of dogs with AD were sensitized to housedust mites, suggesting that Group 2 antigens of housedust mites may be a major allergen in canine AD.

KEY WORDS: allergen, atopic dermatitis, canine, housedust mite, IgE.

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In recent years, the number of patients with allergic diseases has been increasing in dogs as well as in humans [2, 5, 7, 10, 20]. Immunoglobulin E (IgE) antibodies play an important role in the development of immediate hypersensitivity (type I allergic disease) [7, 10, 20]. Binding of multivalent allergens to specific IgE antibody on mast cells or basophils leads to the release of histamine and arachidonic acid metabolites. These substances act as inflammatory mediators inducing various symptoms of allergic diseases such as dermatitis, urticaria, asthma, rhinitis, and conjunctivitis. Housedust mites, *Dermatophagoides pteronyssinus* (*D. pteronyssinus*) and *Dermatophagoides farinae* (*D. farinae*), have been recognized as important environmental allergens in human allergic diseases [19]. The antigenic proteins of housedust mites have been determined with intra-dermal skin test (IDST) and immunochemical method. In fact, over 10 different allergens of housedust mites are identified and characterized with known cDNA and/or N-terminal amino acid sequence [8, 14, 15, 22]. Group 1 and Group 2 antigens are the major allergen in human allergic diseases involved in the onset of type I hypersensitivity [8, 15, 22]. Also in dogs, housedust mites have been recognized as important allergens in the atopic dermatitis (AD) [11, 13, 17], however little is known about the antigenic proteins in housedust mites. And also, it has been reported that Group 1 and Group 2 antigens were rather minor allergen in dogs [13]. Until recently, unlike to human, the involvement of IgE antibody and causative antigens has not been investigated in canine AD.

In the present study, we determined serum levels of IgE antibodies against housedust mites for 165 dogs suffering from AD, and investigated the involvement of housedust mites in canine AD. Furthermore, the reactivity of house-

dust mites-specific IgE antibodies was examined in the dogs sensitized to housedust mites with immunoblot assay and ELISA for the antigenic proteins in housedust mites.

MATERIALS AND METHODS

Antigens: Crude antigens; GS mites mix (mixture of *D. pteronyssinus* and *D. farinae*), was purchased from Greer Laboratory (Lenoir, NC, U.S.A.). Purified antigens; Der f 1 and Der f 2 were purchased from Asahi Beer (Tokyo, Japan), and Der p 1 and Der p 2 were kindly gifted from Dr. Sakaguchi of National Institution of Infectious Disease (Tokyo, Japan).

Dogs: 165 dogs suffering from AD were selected as the clinical cases, which visited or hospitalized at 74 facilities in Japan from April to August in 1996. The clinical diagnosis of AD was performed in accordance with Williemse's criteria [20]. Dogs associated with deep pustular pyoderma and seborrheic dermatitis were excluded from this study.

IMMUNODOT assay: Serum levels of IgE antibody against 25 kinds of allergens were determined with commercially available kits, IMMUNODOT (CMG/Heska Allergy Products, Fribourg, Switzerland) as previously described [3]. Briefly, serum samples were added to the nitrocellulose strips on which 5 allergens were dotted. After incubation, strips were incubated with a peroxidase-labeled mouse monoclonal anti-dog IgE antibody for 2 hr at room temperature. The strips were then incubated with substrate solution (4-chloro-1-naphthol+H₂O₂) for colorimetric detection. The color intensity of each dot was measured with a densitometer (FAG, Fribourg, Switzerland). If the optical density was higher than 0.02, it was considered as positive.

SDS-PAGE and immunoblotting: Crude extracts of

housedust mites were separated by SDS-PAGE according to the method of Laemmli [9] using a 5–20% gradient gel (Atto, Tokyo, Japan) under reducing condition and transferred to nitrocellulose membrane (Millipore, Bedford, MA, U.S.A.). The membrane was incubated with 5% BSA for 1 hr at room temperature and then incubated with the undiluted serum overnight at 4°C. The membrane was further incubated with horseradish peroxidase (HRP)-conjugated mouse anti-dog IgE antibody (CMG/Heska Allergy Products, Fribourg, Switzerland) or HRP-conjugated mouse anti-Der f2 (Asahi Beer, Tokyo, Japan) for 1 hr at room temperature. After incubation, immunoreactive proteins on the membrane were visualized with a TMB Membrane Peroxidase Substrate System (KPL, MD, U.S.A.) according to the manufacturer's protocol.

Enzyme-linked immunosorbent assay (ELISA): Each well of a microtiter plate (MaxiSorp, Nunc, Denmark) was coated with 1 µg of antigen in 0.1 M carbonate-bicarbonate buffer (pH=9.6) overnight at 4°C. After blocked with 5% BSA for 1 hr at room temperature, serum samples at a dilution of 1:20 were added to the wells and incubated for 2 hr at room temperature. The plates were then incubated with a peroxidase-conjugated mouse anti-canine IgE antibody (CMG/Heska Allergy Products, Fribourg, Switzerland) for 2 hr at room temperature. Finally, TMB Membrane Peroxidase Substrate System (KPL, MD, U.S.A.) was added to the well for color development. After the enzyme reaction was stopped with 1 M H₃PO₄, the optical density was measured at 450 nm with a microplate reader (Tosoh, Tokyo, Japan). If the optical density was higher than "mean +3SD" of 10 reference sera, it was considered as positive.

RESULTS

Housedust mites-specific IgE antibody: By using 165 dogs suffering from AD, serum levels of immunoglobulin E (IgE) antibody against 25 allergen including housedust mites were determined. Housedust mites were the most frequent allergen against 90 dogs sensitized (54.5%), followed by storage mites (18.6%), rye grass (13.4%), cat flea (12.5%) and Japanese cedar (10.3%). The frequency of food and mould allergens was comparatively lower than that of indoor or outdoor allergens in this study.

Background factor of 90 dogs sensitized with housedust mites: We investigated the background factor (sex, onset age, season to develop symptoms, keeping place) of 90 dogs sensitized with housedust mites. 34 (37.8%) were male and 57 (62.2%) were female. The onset age included 0–3 years (66.7%), 4–6 years (23.3%), and 7–9 years (10.0%). In the season to develop symptoms of AD, whole year was the highest (63.3%), followed by spring (30.0%), summer (22.2%), autumn (8.9%), and winter (1.1%). In the keeping place, 66 (73.3%) were indoor and 15 (16.7%) were outdoor.

Immunoblotting assay for housedust mites: By using 90 dogs sensitized with housedust mites, we performed immunoblotting assay for crude housedust mites extract in order

Fig. 1A. Immunoblotting patterns showing the reactivity of IgE antibody to housedust mites in different dogs sensitized with them. The crude extracts of housedust mites were separated by SDS-PAGE and the separated proteins were transferred to nitrocellulose membrane. The membrane was incubated with serum and then reactive proteins were detected by HRP-labeled anti-dog IgE antibody. Arrows indicate the frequent proteins against which the patient's IgE antibody or HRP-labeled anti-Der f2 antibody reacts.

to investigate the antigenic proteins in it, that were recognized by patient's IgE antibody (Fig. 1). Figure 1A showed that the molecular mass of antigenic proteins recognized by IgE antibody was ranged from 15-kD to 170 kD. In the experiments, the proteins with the apparent molecular masses of 15, 26, 76, 90, 98, and 170-kD were prominently observed, and the 15-kD was the most dominant among them (Fig. 1B). Since the 15-kD protein was also detected by using anti-Der f2 antibody (Fig. 1A), the apparent molecular masses of the 15-kD might be identical to Group 2 antigen that is a major allergen of housedust mites in human allergic diseases.

ELISA assay for crude and purified antigens of housedust mites: To validate the IgE-reactivity to Group 2 antigens (Der f2 and Der p2), ELISA with purified Der f2 and Der p2 were performed in the 90 dogs sensitized with housedust mites. At the same time, we examined the IgE-reactivity to Group 1 antigens (Der f1 and Der p1), in order to compare the reactivity with Group 2 antigens. Table 1 indicated the number of dogs that showed the positive reactivity of IgE against each purified antigen. The number of dogs with IgE-reactivity against Der f1, Der p1, Der f2, and Der p2

Fig. 1B. The number of cases in the 90 dogs sensitized with housedust mites, reacted to each distinct protein detected by the immunoblotting assay.

was 40, 7, 67, and 20, respectively. The frequency of Der f2 (74.4%), Group 2 antigens of *D. farinae* mite, was comparably high. On the other hand, Der p2 (22.2%), Group 2 antigens of *D. pteronyssinus* mite, were less frequent than Der f1 (44.4%), Group 1 antigens of *D. farinae* mite.

DISCUSSION

Until recently, research in dog allergy has been impaired by the lack of a highly specific antibody that recognize dog IgE antibody. De Boer *et al.* have obtained highly specific and purified monoclonal mouse anti dog IgE antibody [1], and a few diagnostic tests based on the monoclonal antibody such as IMMUNODOT have been commercially available [3, 6]. Such reagents would be used in the clinical diagnosis of canine allergic diseases.

Our clinical study on 165 dogs suffering from AD indicated that the prevalence of specific IgE antibody against housedust mites (54.5%) was the highest in the 25 allergens examined. This result suggested that housedust mites might be important causative allergen in the canine AD, as is the similar phenomena in human. In clinical allergy, it is important to identify the allergenic components in each allergen used for diagnostic test and/or immunotherapy. In case of human allergic diseases, housedust mites is considered to become a critical incident of asthma and rhinitis, and more than 10 kinds of allergenic proteins were known. In these proteins, Group 1 (26-kD) and Group 2 antigens (15-kD) were proved to have specific IgE-reaction in majority of human patients suffering from AD or asthma [14]. Our immunoblotting assay demonstrated that IgE-reactivity to

Table 1. The number of dogs sensitized with purified antigen in housedust mite (Der f1, Der f2, Der p1, Der p2)

Species	Antigen	Positive number (rate)
<i>D. farinae</i>	Der f1 (Group 1)	40 (44.4%)
	Der f2 (Group 2)	67 (74.4%)
<i>D. pteronyssinus</i>	Der p1 (Group 1)	7 (7.8%)
	Der p2 (Group 2)	20 (22.2%)

some kinds of distinct proteins with the molecular masses of 15, 26, 76, 90, 98, and 170-kD was prominently detected in dogs sensitized with housedust mites. Among them, 15-kD protein close to Group 2 antigens was the most dominant one (52/90). On the other hand, 26-kD protein close to Group 1 antigens was rather minor one (8/90). Therefore, the 15-kD of antigenic component recognized by 57.8% of dogs sensitized with housedust mites might be considered as a major allergen in canine AD. The other four antigenic proteins with molecular masses of 76, 90, 98, and 170-kD had frequencies below 30%. Though the significance of these proteins of housedust mites in canine AD are not clear at present, high molecular weight proteins, 92, 98, 177-kD, were recently reported as one of the important allergens in the human athmatic patients [4, 18]. They identified and characterized a 98-kD allergen against which greater than 80% of patients allergic to housedust mites showed IgE reactivity. Nori *et al.* reported that IgGd antibody rather than IgE antibody might play an important role in canine atopic dermatitis, and that the 90-kD proteins recognized by IgGd antibodies were one of the important antigens in housedust mites. They also reported that little IgE-response to Group 1 and Group 2 antigens were observed in the dogs suffering from AD, and they were rather the different responses observed in human allergic diseases.

Then, we investigated the IgE-reactivity to purified mites antigens, Der f1, Der f2, Der p1, and Der p2, in order to validate the results shown in immunoblotting study. In our study, 67 of the 90 dogs (74.4%) sensitized with housedust mites had strong IgE-reactivity to Der f2. In agreement with the immunoblotting study, Der f2 was dominant antigen among the four purified proteins. It should be noted that the IgE-reactivity to the each purified antigen from *D. farinae* mite, Der f1 and Der f2, was relatively higher than that of *D. pteronyssinus* mite, Der p1 and Der p2. In the previous studies, it has been reported that the responses of intradermal skin test (IDST) as well as specific IgE-reaction were much dominant for *D. farinae* compared with *D. pteronyssinus* in dogs suffering from AD, nevertheless the geographic distribution of *D. farinae* and *D. pteronyssinus* mites [12, 13, 16, 21, 22]. Our studies using purified antigen confirmed the results from these previous reports, because *D. pteronyssinus* mite is rather predominant species compared with *D. farinae* mite in Japan [16, 22].

In summary, these studies show the importance of housedust mites in canine AD, and suggest that the Group 2 antigens, especially Der f2 of *D. farinae* mite, are one of the

major allergen in AD dogs allergic to housedust mites.

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