

## Measurement for Canine IgE Using Canine Recombinant High Affinity IgE Receptor $\alpha$ Chain (Fc $\epsilon$ RI $\alpha$ )

Toshihiro TSUKUI<sup>1)</sup>, Masahiro SAKAGUCHI<sup>2)\*</sup>, Keigo KURATA<sup>3)</sup>, Sadatoshi MAEDA<sup>4)</sup>, Keitaro OHMORI<sup>5)</sup>, Kenichi MASUDA<sup>6)</sup>, Hajime TSUJIMOTO<sup>7)</sup> and Shigehiro IWABUCHI<sup>1)</sup>

<sup>1)</sup>Nippon Zenyaku Kogyo Co., Ltd., 1-1 Tairanoue Sasagawa, Asaka-machi, Koriyama, Fukushima 963-0196, Japan

<sup>2)</sup>Department of Veterinary Microbiology, School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan

<sup>3)</sup>Institute of Tokyo Environmental Allergy, 2-2-4 Yushima, Bunkyo-ku, Tokyo 113-0034, Japan

<sup>4)</sup>Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu, 501-1193, Japan

<sup>5)</sup>Laboratory of Veterinary Molecular Pathology and Therapeutics, Division of Animal Life Science, Graduate School, Institute of Symbiotic Science and Technology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan

<sup>6)</sup>Animal Allergy Clinical Laboratories, Inc., 5-4-30 Nishihashimoto, Midori-ku, Sagamihara Kanagawa 252-0131, Japan

<sup>7)</sup>Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

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**ABSTRACT.** To detect allergen-specific IgE in dogs with allergic diseases, we developed a recombinant canine high affinity IgE receptor  $\alpha$  chain (Fc $\epsilon$ RI $\alpha$ )-based IgE detection system. Using the recombinant protein of canine Fc $\epsilon$ RI $\alpha$  expressed by an *Escherichia coli* expression system, we could detect house dust mite (*Dermatophagoides farinae*) allergen-specific IgE in sera from dogs naturally and experimentally sensitized to this allergen with ELISA and western blotting. The IgE binding activity of recombinant canine Fc $\epsilon$ RI $\alpha$  on ELISA was impaired by heat treatment of these sera. The specificity of this recombinant canine Fc $\epsilon$ RI $\alpha$ -based IgE detection system was confirmed by inhibition assays with canine IgE. The recombinant canine Fc $\epsilon$ RI $\alpha$ -based IgE detection system established in this study offers an alternative tool to measure allergen-specific IgE in dogs.

**KEY WORDS:** atopic dermatitis, canine, Fc $\epsilon$ RI $\alpha$ , IgE, mite.

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Canine atopic dermatitis (CAD) is well-recognized as a chronic inflammatory skin disease in dogs [11]. The disease is characterized by pruritis and skin lesions that include erythema, hair loss, hyperpigmentation, and lichenification which are primarily localized on the ventral abdomen, thigh, and/or axillary regions [22]. Previous investigations concerning the etiology of allergy in animals provide evidence for a pathogenesis that closely parallels human allergic disease, the major difference being the ultimate manifestation of disease symptoms [13, 14]. CAD is known to be an immediate type hypersensitivity disease caused by sensitization to various environmental allergens and subsequent exposure to the allergens [2, 4]. Allergens responsible for provoking CAD can be derived from a multitude of sources which include house dust mites, plants, arthropods, epithelia, foods and molds [11, 15, 18].

*In vitro* assays for allergen specific IgE are a convenient and reproducible alternative to intradermal skin testing (IDST) in dogs and the results of such tests can be used to

support a diagnosis of atopic dermatitis and to define appropriate allergens for inclusion in an immunotherapeutic regime [4, 5, 22]. Multiple *in vitro* assays for detection of allergen-specific IgE are available in the veterinary arena [9, 12, 16, 17, 20, 23, 26], yet there is a paucity of useful data describing the assays and comparing the available tests. The best characterized assays for detection of allergen-specific IgE in dogs are a monoclonal antibody cocktail-based ELISA for dog specific IgE [17] and a human high affinity IgE receptor based assay for dog IgE [6, 27]. When directly compared, the concordance of results for these 2 assays was demonstrated to be approximately 92% which is similar to the intra-ELISA/inter-laboratory concordance of results [17]. Generation of high affinity IgE receptor specific for dogs might represent an improvement in reagents available for detection of antigen-specific IgE in dogs.

In the present study we describe the expression, purification, and biotinylation of canine high affinity IgE receptor and provided preliminary information on its utility in an allergen-specific IgE ELISA as well as immunoblotting techniques.

### MATERIALS AND METHODS

*Dogs naturally sensitized with mite allergen:* All dogs used in this study were confirmed to be free of ectoparasite

\*CORRESPONDENCE TO: SAKAGUCHI, M., Department of Veterinary Microbiology, School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan.  
e-mail: sakagum@azabu-u.ac.jp

infestations and cutaneous bacterial infections. This study included 19 dogs that were diagnosed as having historical and clinical findings consistent with CAD [29] and 10 dogs that were known to be negative for CAD and subsequently shown to be non-reactive to mite allergens when evaluated using IDST procedures [19].

**Laboratory dogs artificially immunized with mite allergen:** Five 6-month old beagles were immunized subcutaneously with 100  $\mu$ g of crude *Dermatophagoides farinae* allergen extract (Greer Laboratories, Lenoir, NC, U.S.A.) admixed with 50 mg of alum. The subcutaneous injection was carried out twice with a 2-week interval. After immunization, IDST was performed to confirm sensitization to *D. farinae* allergen extract and recombinant Der f 2 (Seikagaku Corporation, Tokyo, Japan).

**Purification of recombinant canine Fc $\epsilon$ R1 $\alpha$ :** A cDNA clone encoding canine Fc $\epsilon$ R1 $\alpha$  was kindly provided by Dr. Ryo Goitsuka (Science University of Tokyo, Noda, Japan) [8]. The canine Fc $\epsilon$ R1 $\alpha$  cDNA was ligated into EcoRI-XhoI sites of pGEX-4T-1 (GE Healthcare, Piscataway, NJ, U.S.A.) to express canine Fc $\epsilon$ R1 $\alpha$  as a fusion protein with glutathione S-transferase (GST), using a commercially available kit (DNA Ligation kit Ver.2, Takara-Bio, Shiga, Japan). The expression vector, pGEX-cFc $\epsilon$ R1 $\alpha$ , was prepared in *Escherichia coli* competent cells (Top10: Invitrogen Corp, Carlsbad, CA, U.S.A.) and purified using a commercially available kit (Qiagen Endotoxin Maxi kit, Qiagen, Chatsworth, CA, U.S.A.). The plasmid was then introduced into *E. coli* strain BL21 (Novagen, Madison, WI, U.S.A.). The expression of fusion protein was induced by adding 5 ml of 100 mM isopropyl- $\beta$ -D-thiogalactopyranoside (final concentration=1.0 mM). The incubation of culture was continued for an additional 3 hr. Cells pellets were sonicated in phosphate-buffered saline (PBS). Solubilized bacterial suspensions were centrifuged at 10,000 g at 4°C for 10 min, and the supernatant was rotated with glutathione Sepharose 4B (GE Healthcare) at 4°C overnight. The fusion protein combined with glutathione Sepharose 4B was then separated from the supernatant by the addition of reduced glutathione buffer (10 mM glutathione, 50 mM Tris-HCl; pH 8.0). Biotinylation of Fc $\epsilon$ R1 $\alpha$  was performed as described by Nerurker *et al.* [21].

**Detection of canine IgE by ELISA using canine Fc $\epsilon$ R1 $\alpha$ :** The binding activity of recombinant canine Fc $\epsilon$ R1 $\alpha$  to canine IgE was assayed by ELISA. Briefly, microtitre plates (MaxiSorp F96; Nunc, Roskilde, Denmark) were coated with monoclonal canine IgE and purified canine IgG (Bethyl Laboratories, Montgomery, TX, U.S.A.) at concentrations ranging from 0.625 to 1,000 ng/ml in carbonate buffer (pH 9.6), and then blocked with 1% bovine serum albumin (BSA) in PBS at 37°C for 1 hr. For evaluation of binding, biotinylated Fc $\epsilon$ R1 $\alpha$  (1  $\mu$ g/ml) was added to each well and incubated for 2 hr. After washing, peroxidase-conjugated streptavidin (2  $\mu$ g/ml) (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) was added to each well and incubated for 1 hr. Following final washing, the plate was developed with the use of 2, 2'-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS; Sigma Chemical Co., Ltd., St. Louis, MO, U.S.A.) in 0.1 M sodium citrate (pH 4.5). The optical density

(OD) of each well of plates was read at 415 nm with a microplate reader (model 3550; Bio-Rad Laboratories, Hercules, CA, U.S.A.).

**Detection of allergen-specific IgE by ELISA:** Microtiter plates were coated with *D. farinae* allergen extract (Greer Laboratories) (400 ng/ml) in carbonate buffer (pH 9.6), then blocked with 1% BSA in PBS at 37°C for 1 hr. After washing, the individual serum samples of naturally sensitized or healthy dogs were added to each well and incubated for 2 hr. After washing, diluted biotinylated Fc $\epsilon$ R1 $\alpha$  was added to each well and incubated for 2 hr. The remaining allergen-specific IgE ELISA procedures were the same as described above.

**Heat inactivation of IgE in dog sera:** To assess the heat lability of the binding activity of canine IgE for Fc $\epsilon$ R1 $\alpha$ , the sera of 5 naturally *D. farinae*-sensitized dogs and 5 artificially *D. farinae*-immunized dogs were heated at 56°C for 1 hr. Dilutions of the unheated and heat-treated sera were then added to plates coated with *D. farinae* extract (2  $\mu$ g/ml). Mite allergen-specific IgE in the intact and heat-inactivated sera were assayed by ELISA in the above-mentioned ELISA.

**Detection of allergen-specific IgE by western blotting analysis:** Reduced recombinant Der f 2 (1  $\mu$ g/ml) (Asahi Breweries, Tokyo, Japan) was electrophoresed by SDS-PAGE and then electrotransferred to PVDF membrane (GE Healthcare). After blocking with PBS containing 5% non-fat dried milk, the membrane was incubated for 2 hr with naturally Der f 2-sensitized dog sera diluted 1:10 in PBS containing 0.1% Tween-20 and 5% non-fat dried milk. After washing, biotinylated recombinant canine Fc $\epsilon$ R1 $\alpha$  (2  $\mu$ g/ml) was added to the membrane and incubation was continued for 2 hr. Following an additional wash, appropriately diluted streptavidin-HRP was added to the membrane and incubation was continued for an additional 1 hr. Following a final wash, the membrane was incubated with ECL reagent (GE Healthcare) for 5 min and exposed to a radiographic film for 5–30 sec.

For inhibition analysis, recombinant Der f 2 was electrophoresed by SDS-PAGE and then electrotransferred to PVDF membrane. The membrane was incubated with sera of naturally Der f 2-sensitized dogs. After the incubation of Der f 2-sensitized serum with the membrane, admixtures of biotinylated recombinant canine Fc $\epsilon$ R1 $\alpha$  with canine IgE (1  $\mu$ g/ml) or with canine IgG (10  $\mu$ g/ml) were added on the membrane. Detection of canine IgE by recombinant canine Fc $\epsilon$ R1 $\alpha$  was performed using the same method as described for western blotting.

**Statistical analysis:** Statistical analysis was performed using the Student's *t*-test; *P*-values of less than 0.05 were considered significant.

## RESULTS

**Purification of recombinant canine Fc $\epsilon$ R1 $\alpha$ :** The *E. coli* strain BL 21 transformed with the pGEX-cFc $\epsilon$ R1 $\alpha$  produced an extracellular domain of canine Fc $\epsilon$ R1 $\alpha$  (1–172 a.a.) as a fusion protein with GST, and it was isolated by glutathione affinity chromatography. The molecular weight of canine

FcεRIα fused with GST was approximately 40 kDa. The total cell lysate of *E. coli* BL21 transformant and the purified recombinant protein were electrophoresed by SDS-PAGE. A single band at a molecular weight of approximately 40 kDa was detected in the lane of purified recombinant canine FcεRIα (Fig. 1).

**Analysis of binding activity of recombinant canine FcεRIα to canine IgE:** The results presented in Fig. 2 demonstrate the immunoglobulin specificity of the affinity purified recombinant canine FcεRIα. When excess biotinylated recombinant canine FcεRIα was incubated with wells coated with varying concentrations of dog IgE the signal that was observed decreased in an antigen dose dependent fashion. However, when varying concentrations of dog IgG were evaluated, no signal was evident at any of the concentrations tested thereby demonstrating that recombinant canine FcεRIα does not react with IgG immunoglobulins.

**Detection of *D. farinae* allergen-specific IgE by using of recombinant canine FcεRIα on ELISA:** To evaluate the utility of recombinant canine FcεRIα-based ELISA for specific IgE to *D. farinae*, we measured the specific IgE in the sera of 19 dogs with CAD. Using a recombinant canine FcεRIα based ELISA, *D. farinae* allergen-specific IgE was detected in the sera of all the dogs diagnosed with CAD and subsequently shown to be IDST-positive (Fig. 3). In contrast, no *D. farinae*-specific IgE was detectable in the sera of IDST-negative dogs.

**Heat inactivation of IgE in dog sera:** To examine further the specificity of canine FcεRIα binding to canine IgE, serum samples from naturally sensitized (Fig. 4A) as well as artificially (Fig. 4B) immunized dogs were evaluated using our canine FcεRIα based direct bind ELISA specific for mite allergens. The results presented in Fig. 4 document that all dog sera contained *D. farinae*-specific IgE. Although the

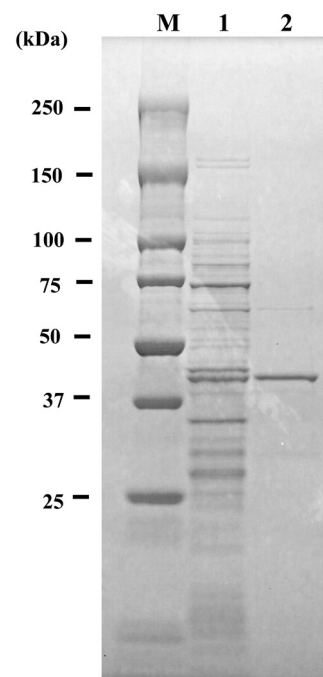


Fig. 1. Expression and purification of recombinant canine FcεRIα in *E. coli*: The DNA encoding extracellular domain of canine FcεRIα was expressed as a fusion protein with GST in pGEX-cFcεRIα. The fusion protein was purified by glutathione affinity chromatography. Molecular weight markers (M), Lane 1: total cell lysate of *E. coli* BL21 transformant (equivalent to 100  $\mu$ l of the culture), Lane 2: recombinant canine FcεRIα fusion protein purified as a fusion with GST.

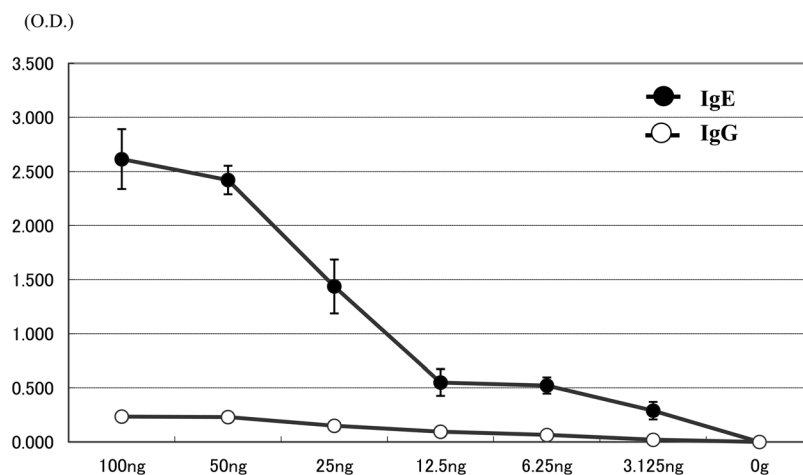


Fig. 2. Measurement of binding activity to recombinant canine IgE and IgG by use of recombinant canine FcεRIα. Both canine IgE (Bethyl Laboratories, Montgomery TX, U.S.A.) and IgG were diluted to concentrations ranging from 31.2 to 1,000 ng/ml. The binding activity of recombinant canine FcεRIα was assayed by ELISA. OD: optical density.

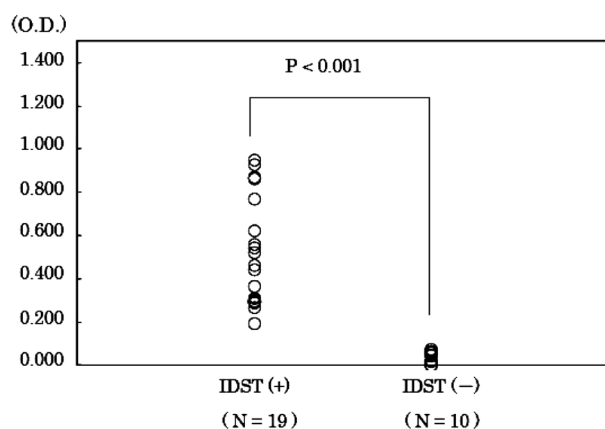


Fig. 3. Measurement of *D. farinae* allergen-specific IgE by ELISA. Sera of dogs with AD (N=19) and healthy dogs (N=10) were assayed by recombinant canine FcεRIα-based ELISA. The OD cutoff value was determined as the mean + 3 X SD of OD obtained from the healthy dogs' sera.

magnitude of response evident in the different samples varied, the reactivity evident in samples heated at 56°C for 1 hr was dramatically reduced to levels near or indistinguishable from negative sample responses. Important to this evaluation is the observation that heated negative serum samples were unaffected by the heating process; signals remained indistinguishable from background responses and no increase in non-specific reactions are evident.

**Recombinant canine FcεRIα binding to *D. farinae*-specific IgE in western blotting:** The utility of recombinant canine FcεRIα in western blotting analysis was evaluated. Recombinant Der f 2 was electrophoresed by SDS-PAGE and then electrotransferred to PVDF membrane. The serum from a dog diagnosed with CAD and identified as Der f 2-sensitized by IDST was added to the membrane, and Der f 2-specific IgE was detected with biotinylated recombinant canine

FcεRIα (Fig. 5, lane 1). To examine the IgE-specificity of this western blotting reagent, we adsorbed the biotinylated recombinant FcεRIα with purified canine IgE before adding to the membrane. The Der f 2-specific IgE signal evident with unadsorbed recombinant FcεRIα (Fig. 5, lane 1) was completely eliminated when biotinylated recombinant canine FcεRIα was adsorbed with purified canine IgE (Fig. 5, lane 2). In contrast, a signal of equal intensity to unadsorbed recombinant FcεRIα was evident when biotinylated recombinant canine FcεRIα was adsorbed with purified canine IgG (Fig. 5, lane 3). These results provide additional support for the IgE specificity of recombinant canine FcεRIα.

## DISCUSSION

FcεRI is an oligomeric protein that is expressed on the surface of mast cells and basophils as a tetrameric complex consisting of one α, one β, and two γ subunits [1]. The FcεRIα binds to the Fc domain of IgE with high affinity; consequently, the FcεRI complex plays an important role in triggering allergic responses [1]. Expression and purification of the extracellular domain of human FcεRIα using a variety of systems has been reported. Chinese hamster ovary cell (CHO) transfectants [3], baculovirus-infected Sf-9 cells, yeast transformants [30], and *E. coli* transformants [28] have all been used to produce recombinant human FcεRIα. The utility of this molecule in detecting antigen-specific IgE in human sera as well as a variety of other animals has been well documented. In fact, one of the characterized commercial ELISA for allergen specific IgE in dogs [27] incorporates a human high affinity FcεRIα. However, Hakimi *et al.* reported that human FcεRIα exhibited lower affinity for rat IgE when compared to the affinity for human IgE [10] and it is likely that lowered cross species affinity exists for human FcεRIα in other animals as well. Therefore, successful expression, purification and characterization of a high affinity canine FcεRIα might represent a desirable alternative for assays intended for detecting antigen specific IgE in dogs.

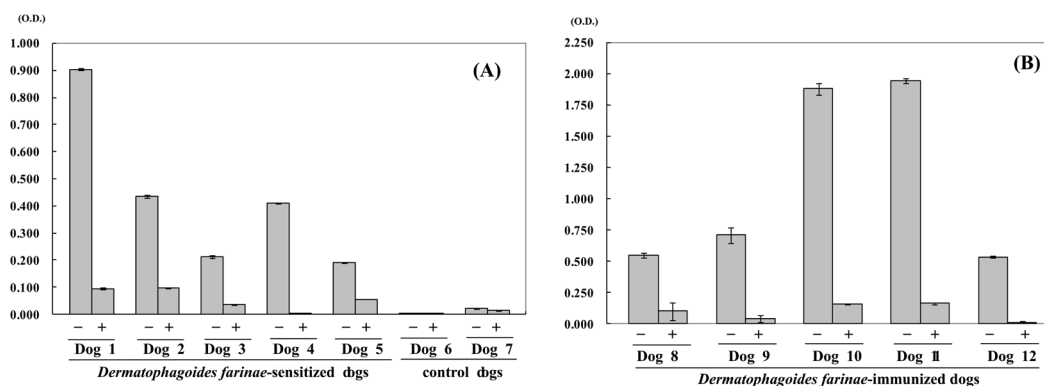


Fig. 4. Detection of *D. farinae* allergen-specific IgE and binding activity of recombinant canine FcεRIα in ELISA before and after heat inactivation. (A) Sera of *D. farinae*-sensitized dogs (Dog 1 to Dog 5) and healthy dogs used as controls (Dog 6 and Dog 7) and (B) Intact (-) and heat inactivated (+) sera of *D. farinae*-immunized dogs (Dog 8 to Dog 12) were assayed at 56°C for 1 hr by using recombinant canine FcεRIα.

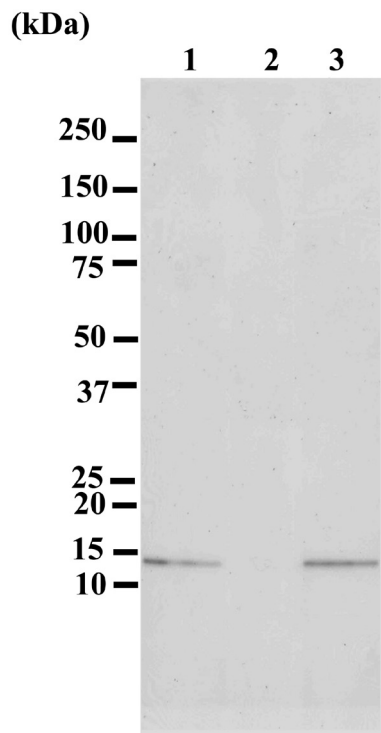


Fig. 5. Detection and inhibition of binding activity to Der f 2-specific IgE by western blotting: Der f 2 (15 kDa) was electrophoresed by SDS-PAGE and then electrotransferred to PVDF membrane. The serum of a naturally Der f 2-sensitized dog was assayed. Lane 1: detection with recombinant canine FcεRIα, Lane 2: detection with mixture of recombinant canine FcεRIα and canine IgE (1 μg/ml), Lane 3: detection with mixture of recombinant canine FcεRIα and canine IgG (10 μg/ml).

The recombinant canine FcεRIα used in the present study was expressed with GST. The commonly used GST gene fusion system [25] allowed us to successfully and more easily purify recombinant canine FcεRIα from *E. coli* lysate. Attempts to yield purified FcεRIα as a single protein containing only the extracellular domain of canine FcεRIα and free of GSTs were unsuccessful. Nonetheless, the experiments conducted in this study demonstrate that the GST-FcεRIα fusion protein exhibits specific binding activity to canine IgE and confirms that GST has little influence on the application of FcεRIα for the detection of canine IgE. Whether or not the affinity of the canine GST-FcεRIα fusion protein is affected by the GST remains to be determined.

The specificity of recombinant canine FcεRIα binding to canine IgE was confirmed using three different approaches. Similar to the evaluation of human FcεRIα [27], we demonstrated IgE isotype specificity for the canine FcεRIα using ELISA for solid phase bound purified canine IgE and purified canine IgG. Our purified canine FcεRIα bound to canine IgE in an antigen dose dependent fashion that showed

reactivity at concentrations as little as 3 ng, but reactivity of the purified canine FcεRIα to canine IgG was completely lacking even at a concentration of 100 ng. The utility of recombinant canine FcεRIα in western blotting analysis was confirmed using recombinant house dust mite allergen (Der f 2) and sera from mite reactive dogs. Further, Der f 2-specific IgE could not be detected using western blotting methods with biotinylated FcεRIα that was adsorbed (inhibition) with purified canine IgE. In contrast, adsorption with purified canine IgG did not interfere with immunoblot detection of Der f 2-specific IgE antibodies. Finally, mite reactivity evident in dog serum using an allergen-specific IgE ELISA that incorporates biotinylated canine FcεRIα was dramatically reduced (>80%) following heat treatment of the sera for 1 hr at 56°C. Such reduction in signal is consistent with the known heat lability of IgE [17, 24]. Equally important is the lack of reactivity in heated samples, which also contains very high levels of heat stable mite specific IgG (data not shown) thereby confirming the lack of canine FcεRIα reaction with IgG.

Although the performance characteristics of this detection reagent in an all encompassing allergen-specific IgE ELISA remains to be determined, it is logical to assume that its performance in detecting allergen-specific IgE in dog sera will parallel and exceed the performance characteristic of the human FcεRIα currently being used [27]. In essence, the FcεRIα behaves as a single epitope detecting tracer molecule. In this regard, its detection capability is akin to that of the Fab portion of monoclonal antibodies and as such might represent a good adjunct to currently available monoclonal cocktail based ELISA for allergen-specific IgE in dogs [17]. In fact, admixture of canine high affinity FcεRIα with high affinity canine IgE-specific monoclonal antibodies will likely yield an assay with increased sensitivity while maintaining specificity [7]. Continued development and characterization of such a detection system will undoubtedly result in the next generation of canine allergen-specific IgE detection ELISA and immunoblotting detection reagents.

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