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Research paper

Establishment of a quantitative ELISA for the measurement of allergen-specific IgE in dogs using anti-IgE antibody cross-reactive to mouse and dog IgE

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ABSTRACT

As IgE plays a pivotal role in type I hypersensitivity-mediated allergic diseases, it is valuable to measure absolute quantity of serum antigen-specific IgE for clinical and research purposes. Here we describe a novel ELISA system that enables quantification of antigen-specific IgE in ng/ml in dogs. A newly developed monoclonal antibody (CRE-DM) was shown to recognize canine and mouse IgE equally in a dose dependent manner, but it did not recognize canine IgG. The reactivity of CRE-DM to canine IgE was also confirmed by an inhibition ELISA using canine IgE as an inhibitor and the maximum inhibition rate was 91.3%. In order to know whether canine IgE specific to an allergen could be quantitatively measured with an ELISA using CRE-DM, we established a quantitative ELISA that could measure canine IgE recognizing Cry j 1, one of the major allergens of Japanese cedar pollen. In this ELISA, a standard curve was created by using concentration-predetermined Cry j 1-specific monoclonal mouse IgE. According to the standard curve, the concentration of Cry j 1-specific IgE in dogs that were experimentally sensitized to Japanese cedar pollen could be calculated and determined in ng/ml. The specificity of the Cry j 1-specific IgE ELISA using CRE-DM was also confirmed by inhibition ELISA using canine IgE as an inhibitor and the inhibition rate was 97.0%. Reproducibility of the ELISA in three independent assays was determined using groups of pooled canine sera whose Cry j 1-IgE titers ranged from 155.9 to 888.2 ng/ml. Intra- and inter-assay reproducibility was determined with coefficient of variation ranging between 3.1–5.2% and 2.2–8.0%, respectively. These results demonstrated that the ELISA utilizing CRE-DM was a specific, reliable and robust new laboratory test that could quantify absolute amount of antigen-specific IgE in canine serum. The ELISA will serve as a useful tool in the clinics to evaluate the change of serum IgE titers during anti-allergic treatments as well as during seasonal fluctuation of allergen exposure.

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1. Introduction

IgE plays a pivotal role in provoking IgE-mediated allergic reaction and inducing various allergic diseases such

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as canine atopic dermatitis (AD) and cutaneous adverse food reactions in dogs. The relationship between IgE and allergic diseases has been extensively investigated in dogs. Allergen-specific IgE has been known to initiate degranulation of the basophils in the skin of experimental flea allergy model dogs (Halliwell and Schemmer, 1987). It was also shown that the basophils of the peripheral blood of dogs with AD (Masuda et al., 2000) and food allergy (Ishida et al., 2003) are initiated to release histamines through antigen-IgE mediated stimulation. The serum titer of Japanese cedar pollen was reported to fluctuate in parallel to the manifestation of allergy-related clinical signs and the pollination season (Masuda et al., 2002). An oral exposure to food antigens in dogs with food allergy induced clinical signs, which were accompanied by the increase of allergen-specific IgE in the sera (Jackson et al., 2003). Because of the accumulating evidence suggesting the involvement of IgE in the pathogenesis of IgE-mediated allergic diseases in dogs, it is becoming quite important to detect allergen-specific IgE in clinical cases of canine AD and food allergy for diagnosis and therapeutic purposes (DeBoer and Hillier, 2001a).

Two major test methods have been used in dogs to detect allergen-specific IgE; intradermal test (IDT) and serum antigen-specific IgE test (serum IgE test). IDT is the *in vivo* test to see whether wheals are formed at the sites of intradermal injection of allergens. When a positive immediate reaction to an allergen solution is detected in IDT, it can be concluded that the patient has an IgE-mediated hypersensitivity to that allergen, thus supporting the diagnosis of IgE-mediated allergic disease. IDT has been popularly used in veterinary practice (Hillier and DeBoer, 2001), but however, there are some limitations for its clinical use. Because of the nature of this test as a qualitative method, the test results are only obtained as negative or positive. Therefore, it is difficult for clinicians to understand the magnitude of sensitization and to monitor the therapeutic efficacies using IDT results. It is also reported that IDT is prone to false negative results because the sufficient allergen concentrations that give the proper test results differ from allergen to allergen and from patient to patient (Hensel et al., 2004). Moreover, IDT is also reported to exhibit false positive reactions due to sub-clinical hypersensitivity (Pastorello, 1993; Codner and Tinker, 1995). In addition, because IDT requires particular procedures such as intradermal injections, antigen preparation and animals to be immobilized during the test, IDT is inconvenient for veterinary practitioners who are not specialized in allergic diseases to perform as a routine clinical test.

Serum IgE tests have been developed during the last three decades in expectation to overcome the limitations of IDT. Serum IgE tests are the methods to detect allergen-specific IgE in canine sera. Currently in most commercial tests available in the veterinary field, various forms of ELISAs are utilized as assay systems. For the detection of canine IgE, anti-IgE antibodies or recombinant extracellular segments of the human high affinity IgE receptor (rhFcεRIα) are used (DeBoer and Hillier, 2001b). The amount of the detecting reagent bound to IgE is measured by detecting the signals developed by enzyme-mediated methods. Thus the generated signals are deemed to reflect

the amount of IgE in the tested serum. The intensity of signals is further converted into units, scores or grades that are defined by each laboratory and reported to the clinicians. Because the reference reagents and the data processing platforms used in the tests differ for each laboratory, it is difficult to compare results among the different laboratories. Also the results must be regarded as qualitative or semi-quantitative because the results are not given in an absolute quantification such as in ng/ml but in comparative scales. Moreover, there have been continuous debates until now on how to interpret and link the serum IgE test results with clinical findings (DeBoer and Hillier, 2001b; Zur et al., 2002). For these reasons, a truly quantitative serum IgE test has been sought.

In basic immunological research utilizing mice, there are a number of reports that utilize ELISAs which are able to quantify allergen-specific mouse IgE in µg/ml. In these ELISAs, anti-mouse IgE antibodies are used for the detection of IgE and pre-quantified allergen-specific monoclonal mouse IgEs are used to create standard curves. Then, the concentrations of allergen-specific IgE in mouse sera are calculated according to the standard curves. For instance, a quantitative ELISA system for ovalbumin (OVA)-specific mouse IgE was established with a monoclonal mouse IgE recognizing OVA and used to detect the serum titer of OVA-specific IgE at µg/ml in mice (Hamada et al., 2003). As in dogs, monoclonal canine IgE specific to filarial antigen has been developed and reported (Gebhard et al., 1995). However, the IgE described in the literature can only be used as a standard reference to measure the worm-specific IgE, which seems to have little use in allergy testing. In order to establish an ELISA for the measurement of allergen specific IgE in dogs by such systems, it is required to establish monoclonal canine IgE for each allergen to be tested, which seems to be difficult to achieve.

To overcome these issues, we attempted to establish a novel ELISA system that could measure the absolute quantity of antigen-specific serum IgE in dogs by using a newly developed anti-IgE antibody that cross reacts equally to IgEs of dog and mouse (CRE-DM). A monoclonal mouse IgE specific to Cry j 1, one of the major allergens of Japanese cedar pollen, was used as a reference standard to quantify Cry j 1-specific IgE in canine sera. This is the first report to demonstrate the method of an absolute quantification of allergen-specific IgE in ng/ml scale in canine sera.

2. Materials and methods

2.1. Preparation of anti-IgE mAb cross-reactive to canine and mouse IgE

Six female rats (Wistar/ST) were all immunized with 20 µg of mouse IgE (BD Biosciences, San Jose, CA, USA) and canine IgE (Bethyl Laboratories, Montgomery, TX, USA) two times with a 2-week interval. After 4 weeks, the spleen cells were obtained and fused with a mouse myeloma cell line, X63Ag8.653. A hybridoma cell clone that produced an antibody reacting with canine and mouse IgE in the culture supernatant was selected by direct ELISAs using mouse IgE or canine IgE as antigens. The cell clone was expanded and injected intraperitoneally into rats (Wistar/ST) and

ascites was obtained 6 months after the injection. From the ascites, rat IgG was purified with ammonium sulfate precipitation and biotinylated with a kit (EZ-Link, Pierce Biotechnology, Rockford, IL, USA). The IgG concentration was determined with a spectrophotometer at 280 nm. This anti-IgE antibody was supposed to be cross-reactive to IgEs of dog and mouse, thus the antibody was named CRE-DM. All aspects of this experiment were approved by Animal Care and Use Committee of Zenoaq Nippon Zenyaku Kogyo Co., Ltd. (Zenoaq, Fukushima, Japan).

2.2. Evaluation of the reactivity and the specificity of CRE-DM

Reactivity of the CRE-DM to canine IgE, canine IgG and mouse IgE were evaluated by ELISA using biotinylated CRE-DM as a detecting antibody. The microwells were coated with various concentrations ranging from 1 to 100 ng/ml of canine IgE (Bethyl Laboratories), canine IgG (Rockland Immunochemicals, Inc. Gilbertsville, PA, USA), or mouse IgE (BD Biosciences). The wells were blocked with 1% gelatin in PBS for 2 h at room temperature. After washing three times with PBST, 100 μ l of 0.5 μ g/ml biotinylated CRE-DM diluted in dilution buffer (1% gelatin in PBST) was added to each well. After 2-h incubation at room temperature, the wells were washed three times with PBST and 100 μ l of 0.05 U/ml Streptavidin-beta-Gal conjugate (Roche Diagnostics GmbH, Penzberg Germany) in dilution buffer was added to each well and incubated for 2 h at room temperature. The wells were then washed three times with PBST and 100 μ l of 0.1 mM 4-methylumbelliferyl β -D-galactopyranoside (4MU, Sigma–Aldrich, Saint Louis, MS, USA) in reaction buffer (0.1% gelatin, 0.1 M NaCl and 1 mM MgCl₂ in phosphate buffer, pH 7.4) was added. Following 1-h incubation at room temperature, 100 μ l of stop solution (0.25 M Na₂CO₃) was added and detected with microplate reader Gemini XPS (Molecular Devices, Sunnyvale, CA, USA). The data were analyzed using Softmax Pro (Molecular Devices). In the inhibition ELISA assay, which aims to qualify the specificity of the reaction, biotinylated CRE-DM was pre-incubated overnight at 4 °C with various amounts of canine IgE, and the assays were performed similarly as described above.

2.3. Serum samples from experimentally sensitized dogs

To obtain high titer serum used for the measurement and inhibition experiments in Cry j 1-specific IgE ELISA, a dog was experimentally sensitized to Japanese cedar pollen. The sensitization procedure was carried out as previously described (Yamashita et al., 2000). A dog was maintained and the experiment was carried out at Zenoaq. Briefly, a healthy beagle was subcutaneously injected twice at 2-week interval with 100 μ g of Japanese cedar pollen antigen in 3 ml of saline-emulsified alum. Two weeks after the second injection, production of IgE to Japanese cedar pollen antigen was confirmed by IDT (data not shown), and serum sample was collected. All aspects of this experiment were approved by Animal Care and Use Committee of Zenoaq.

To obtain serum of various titers of IgE to Cry j 1, four Maltese-beagle atopic (MBA) dogs were sensitized with Cry j 1 with a combination of intraperitoneal and epicutaneous antigen stimulations. Dogs first received two intraperitoneal injections of 200 μ g of a mixture of Cry j 1 and 2 (ratio: 5:2). Dogs were further sensitized with additional epicutaneous applications of 200 μ g of crude Japanese cedar pollen in mineral oil, with or without tape stripping, on a weekly to monthly basis depending on their allergen-specific IgE serum levels. The serum samples were collected at several time points before and after the sensitization procedure. Successful sensitization was confirmed by the positive reactions to Cry j 1 in IDT, which showed wheal formation comparable to that of the histamine positive control. The sera were used for the quantification of serum Cry j 1-specific IgE pre- and post-sensitization and for determining the reproducibility of the Cry j 1-specific IgE ELISA utilizing CRE-DM. All aspects of the study were approved beforehand by North Carolina State University Institutional Animal Care and Use Committee (IACUC).

2.4. Determination of the optimal dilution rate for the dog serum sample

To address an issue that IgGs in the serum that share reactive antigens with IgEs could possibly influence the reactions between antigens and IgEs in the ELISA, it was considered essential to use the appropriate serum dilution rate to measure antigen-specific IgE in the serum. To determine the optimal dilution rate for serum samples, high Cry j 1-IgE titer serum described above was diluted to various dilution rates ranging from 10 to 100,000 and used in the ELISA. Because the dog was intensely immunized so as to produce large amounts of antibodies, the sample was considered to contain large amounts of IgGs specific for Cry j 1 as well, which should well exceed the amount of IgGs that could possibly be produced in the spontaneous sensitization to the allergen. From these reasons we used this serum as a representative sample. A serum of healthy beagle, obtained from Zenoaq, was diluted simultaneously and used in the assay to serve as a blank control for each dilution rates. Microwells were coated with 100 μ l of 1 μ g/ml Cry j 1 (Hayashibara Biochemical Labs., Inc., Okayama, Japan). Wells were blocked with 1% gelatin PBS for 2 h at room temperature and then washed three times with PBST. Then the diluted samples were applied to wells and incubated overnight at 4 °C. After washing three times with PBST, 100 μ l of biotinylated CRE-DM in dilution buffer was added to each well. The rest of the procedure was carried out as described above.

2.5. Quantification of Cry j 1-specific IgE in canine serum

To determine whether canine IgE specific to an allergen could be quantitatively measured with an ELISA using CRE-DM and Cry j 1-specific monoclonal mouse IgE, we performed a quantitative ELISA specific for canine IgE to Cry j 1. Microwells were coated with 100 μ l of 1 μ g/ml Cry j 1 (Hayashibara Biochemical Labs., Inc.). Wells were

blocked with 1% gelatin PBS for 2 h at room temperature and then washed three times with PBST. Sera from clinical cases were diluted 1:1000 in dilution buffer and 100 μ l were applied to microwells. To create a standard curve, anti-Cry j 1 monoclonal mouse IgE was obtained by a standard method to be used as a standard reference. Briefly, BALB/c mice were immunized twice in a week interval with 5 μ g of Cry j 1 and 2 mg of alum per mouse. Three days after the last immunization, the mice were sacrificed and their splenic cells were fused with P3U1 myeloma cells at 3:1 ratio with polyethylene glycol 4000 (Wako Pure Chemical Industries, Osaka, Japan). The fused cells were seeded in 96 well flat bottom plate at the concentration of 6×10^4 cells per well (splenic cells) and cultured in HAT selection medium (complete RPMI with 10% FCS containing 0.1 mM hypoxanthine, 0.016 mM thymidine 0.4 nM aminopterin) for 10 days. The culture supernatants of growing cells were screened for the presence of mouse IgE with the Cry j 1-IgE ELISA using anti-mouse IgE monoclonal antibody (Yamasa Corporation, Chiba, Japan). Finally, a positive clone was established as a hybridoma secreting IgE specific to Cry j 1.

Obtained anti-Cry j 1 monoclonal mouse IgE was quantified with mouse IgE EIA kit (Yamasa Corporation) and diluted to concentrations ranging 0.156–10 ng/ml and 100 μ l of each solution was applied to wells of ELISA plates. After incubating overnight at 4 °C, wells were washed and 100 μ l of biotinylated CRE-DM diluted in dilution buffer at 0.5 μ g/ml was added to each well. The rest of the procedure was carried out as described above. To further investigate whether CRE-DM could specifically detect IgE in canine serum, CRE-DM was incubated beforehand with canine IgE as an inhibitor for overnight at 4 °C (pre-treated CRE-DM). Inhibition ELISA was performed similarly as described above but replacing CRE-DM with the pre-treated CRE-DM.

2.6. Evaluation of the reproducibility of the ELISA utilizing CRE-DM

The reproducibility of the ELISA utilizing CRE-DM was evaluated by comparing results among the wells in three different plates within an assay (intra-assay) and among the wells of three independent assays which were performed on different days (inter-assay). Canine sera with various titers were prepared from the Cry j 1-sensitized MBA dogs as described above and the concentrations of Cry j 1-specific IgE in each serum were determined beforehand with the ELISA using CRE-DM. According to the concentrations, sera were divided into three groups, high, intermediate and low. Three serum samples were chosen from each group and pooled. On each plate the sera of high, intermediate and low groups were applied to wells. The first assay was carried out with three plates in parallel and the data were used for intra-assay evaluation. The second and third assays were carried out with one plate each. For inter-assay evaluation, data from three plates, each from three independent assays, were collected and analyzed. Coefficient of variation (CV) was calculated as (SD/mean) $\times$ 100 (%) for each sera group.

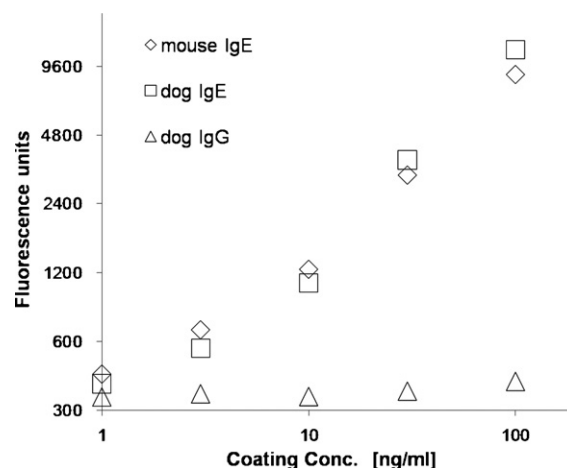


Fig. 1. Reactivity of CRE-DM against canine IgE, canine IgG and mouse IgE. Reactivity of the CRE-DM was evaluated in ELISA by applying the CRE-DM to the wells coated with canine IgE, canine IgG or mouse IgE. Resulting signals are plotted as fluorescence unit. Open diamonds, squares and triangles indicate signals from mouse IgE, canine IgE and canine IgG, respectively. The result is the representative of three independent experiments which gave similar results.

3. Results

3.1. Reactivity and specificity of CRE-DM

The reactivity of CRE-DM to IgE was first determined by the direct ELISA. It was shown that the CRE-DM reacted equally to canine IgE and mouse IgE in a dose dependent manner (Fig. 1). However, the CRE-DM did not react to canine IgG. The reactivity was further analyzed by an inhibition ELISA using canine IgE as an inhibitor. When CRE-DM was pre-incubated with various amounts of canine IgE, the reaction was obviously reduced. The inhibition rate ranged from 83.0% to 91.3% with the inhibitor/CRE-DM ratio varying from 0.5 to 10. The maximum reduction was obtained when the ratio was 10 (Fig. 2). Taken together, the CRE-DM was considered to react specifically to canine IgE and mouse IgE, but not to canine IgG.

3.2. Determination of the optimal dilution rate for the dog serum sample

To determine the serum dilution rate that is optimal for the quantification of Cry j 1-specific IgE in the dog serum, the serum with high IgE titer was diluted into indicated dilution rates and the ELISA was performed (Fig. 3). As the result, the fluorescence units and the dilution rates showed good correlation at the dilution rates between 100,000 and 1000 but plateaued at the dilution rates lower than 300. This was considered to indicate that the reactions between the Cry j 1-specific IgEs and the antigens were inhibited at the dilution rates <300 possibly due to the influence of the Cry j 1-specific IgGs in the serum that shared antigens with IgEs. From these results, we concluded that the dilution rate of 1000 was optimal in the measurement of allergen specific IgE in dog sera.

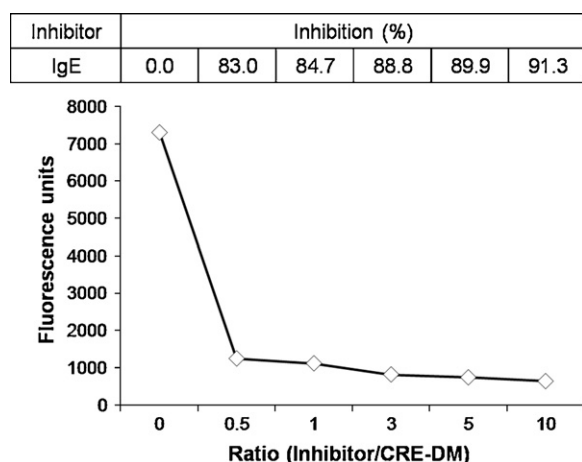


Fig. 2. Inhibition ELISA using canine IgE as an inhibitor. The CRE-DM was pre-incubated prior to ELISA with canine IgE to evaluate the specificity of the reaction. Resulting signals are plotted as fluorescence units with open diamonds. Percentages of the reductions are shown in the table. The result is the representative of three independent experiments that gave similar results.

3.3. Quantification of Cry j 1-specific IgE in canine serum samples

To test whether allergen-specific IgE in canine serum could be measured with an ELISA using CRE-DM, Cry j 1-specific IgE was measured in serum from the dog experimentally sensitized to Japanese cedar pollen. A standard curve in the ELISA was created by using the concentration-predetermined monoclonal mouse IgE specific to Cry j 1. As a result, Cry j 1-specific IgE in the serum could be measured and the concentration was determined as 11951.6 ng/ml. Furthermore, in order to confirm the specificity of this ELISA, an inhibition ELISA was performed with canine IgE as an inhibitor. When canine IgE was used as an inhibitor, the concentration of Cry j 1-specific IgE in the serum was

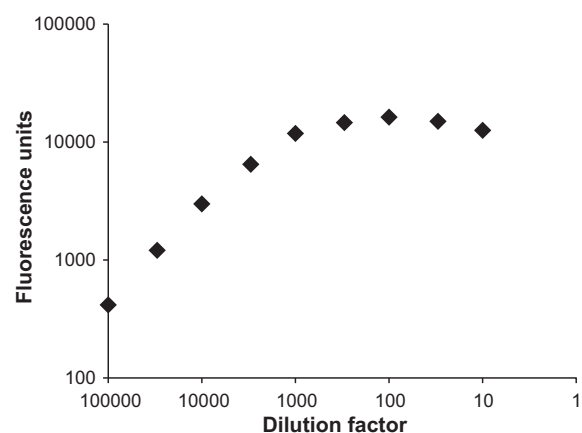


Fig. 3. Determination of the optimal dilution rate for the dog serum sample. The dog serum with high Cry j 1-specific IgE was diluted at various dilution rates as indicated. The resulting signals from each dilution rates are indicated as fluorescence units and plotted with solid diamonds. The result is the representative of two independent experiments that gave similar results.

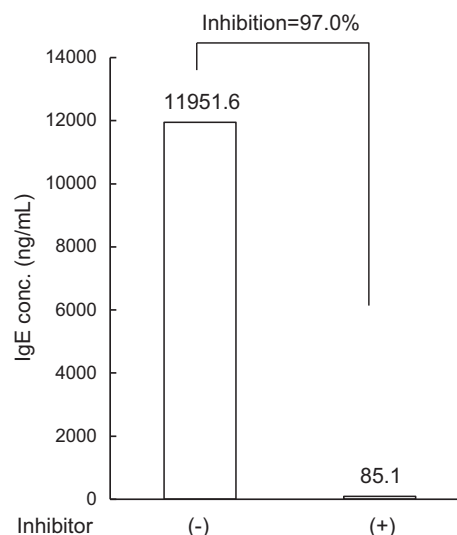


Fig. 4. Cry j 1-specific ELISA utilizing CRE-DM in canine serum samples. The absolute concentration of Cry j 1-specific IgE in canine sera was analyzed. Measurement of Cry j 1-specific IgE in a dog experimentally sensitized to Japanese cedar pollen. Also, the specificity of the ELISA was evaluated by pre incubating CRE-DM with canine IgE as an inhibitor. Inhibition rates are indicated in the figure. The result is the representative of two independent experiments that gave similar results.

reduced to 85.1 ng/ml, which indicated that the inhibition rate was 97.0% (Fig. 4). These data indicated that the reactivity of CRE-DM to canine IgE was maintained in the ELISA for canine serum as samples and allergen-specific IgE concentration in dog serum could be measured. To further confirm that serum antigen-specific IgE could be measured in other dog serum samples with different sensitization statuses, we measured the concentrations of Cry j 1-specific IgE in MBA dogs during pre- and post-sensitization with Cry j 1 (Table 1). The results clearly demonstrated that Cry j 1-specific IgE concentrations increased from nearly undetectable levels before the sensitization to high concentrations such as 544.8–1852.5 ng/ml after the sensitization. From these results, it was concluded that the ELISA utilizing CRE-DM could be used to measure allergen-specific IgE in the serum of dogs hypersensitive to Japanese cedar major allergens.

3.4. Reproducibility of the Cry j 1-specific IgE ELISA utilizing CRE-DM

Using sera from MBA dogs experimentally sensitized to Cry j 1, the reliability of the Cry j 1-specific IgE ELISA was determined by evaluating the reproducibility of the values in pooled sera with the high, intermediate, and low

Table 1
Cry j 1-specific IgE concentration in MBA dogs sensitized with Cry j 1.

Dog no.	Pre-sensitization (ng/ml)	Post-sensitization (ng/ml)
1	15.6	1852.5
2	na ^a	1513.4
3	0	613.1
4	0	544.8

^a Not applicable due to a failure in sample collection.

Table 2

Reproducibility of the Cry j 1-specific IgE ELISA utilizing CRE-DM.

Comparison	Groups	Data1 (ng/ml)	Data2 (ng/ml)	Data3 (ng/ml)	Mean (ng/ml)	CV (%)
Intra-assay	Low	155.9	172.2	168.7	165.6	5.2
	Intermediate	413.4	391.2	388.5	397.7	3.4
	High	841.3	888.2	841.9	857.1	3.1
Inter-assay	Low	172.2	156.0	180.0	169.4	7.2
	Intermediate	391.2	411.6	456.5	419.8	8.0
	High	888.2	887.4	854.2	876.6	2.2

concentrations of Cry j 1-specific IgE in three independent experiments. As the results, the intra-assay CV was found to be 5.3%, 3.4% and 3.1% in the pooled sera of low, intermediate and high IgE groups, respectively. Likewise, the inter-assay CV was 7.2%, 8.0% and 2.2% in the pooled sera of low, intermediate and high IgE groups, respectively (Table 2). These results indicated that the values obtained with the Cry j 1-specific IgE ELISA in this study were highly reproducible and reliable.

4. Discussion

In order to measure an absolute concentration of allergen-specific IgE in canine sera, it is necessary to use a monoclonal antibody that is highly specific to canine IgE. Since there are an excess amount of IgG which share reactive antigen with IgE in the serum (Hill et al., 1995), and that IgE and IgG have high similarities (Patel et al., 1995), IgE measurement will be influenced if the anti-IgE antibody used in the measurement can cross-react to IgG even at a low level. Therefore, an anti-IgE antibody used in the detection of IgE must be highly specific to IgE and required to be confirmed by proper assays so that the antibody does not react to IgG. We first examined the reactivity of the anti-IgE antibody, CRE-DM, especially focusing on the reactivity to canine IgE and IgG. In our study, it was shown that CRE-DM reacted specifically to purified canine IgE and mouse IgE in a dose dependent manner, but not to canine IgG, which was further confirmed by the inhibition ELISA. The specificity of CRE-DM was also confirmed in the ELISA for canine antigen-specific IgE using the serum of an experimentally sensitized dog. Unlike in the other previous studies that evaluated the reactivity of anti-IgE antibody or rhFcεRIα to canine IgE only by heating the serum at 56 °C (Stedman et al., 2001; Lee et al., 2009), we could obtain direct evidence that verified the reactivity of CRE-DM by the combination of ELISA and inhibition ELISA experiments. Also, in a currently ongoing study, we could determine the linear epitope sequence that is recognized by CRE-DM. CRE-DM was found to recognize an epitope located in the CH₃ regions of dog and mouse IgEs that showed high amino acid sequence similarity between both species (Tsukui T., et al., article in preparation). Furthermore, the identical sequence was not found in the amino acid sequence of dog IgG. According to these results, it was concluded that the ELISA using CRE-DM was highly specific for the measurement of canine IgE.

To measure an allergen-specific IgE by a quantitative ELISA, pre-quantified allergen-specific IgE is required as the reference standard for each allergen to be tested. However, the only purified canine allergen-specific IgE reported

so far is against filarial antigen (Gebhard et al., 1995). Alternatively, the pooled serum of clinical cases sensitized to an allergen such as Japanese cedar pollen has been used as a reference in a comparative measurement ELISA whose test results were expressed at units/ml (Masuda et al., 2000). However, in a comparative measurement ELISA, the results must be considered as semi-quantitative and, as a drawback of this method, results cannot be compared among different allergens since the references must differ from assay to assay. In contrast, allergen-specific mouse IgE measurement has been implemented for different allergens by establishing pre-quantified allergen-specific monoclonal mouse IgE as reference standards for each allergen (Hamada et al., 2003). Therefore, the results could be expressed in, for example, µg/ml, which enables the comparison of the amount of allergen-specific IgE among different allergens. With this information, we conceived the objective that we could measure canine allergen-specific IgE by using allergen specific mouse IgE as a reference standard if we had an antibody that could cross-react to both canine and mouse IgE. Therefore, it was a great advantage to obtain an anti-IgE antibody, CRE-DM, which was proven to recognize both canine and mouse IgE in a dose dependent manner. In fact, we could quantify Cry j 1-specific IgE in canine sera at ng/ml by using Cry j 1-specific monoclonal mouse IgE as a reference standard in this study. The established method in this study can be applied to other allergen-specific IgE ELISAs by creating pre-quantified allergen-specific mouse IgE to various allergens. For this purpose, we are currently working on to expand the test system to measure allergen-specific canine IgE to various allergens by establishing reference standards using mouse sera sensitized with different allergens. With such a multi-allergen quantification system, it will become possible to compare the exact amount of allergen-specific IgEs among the different allergens in dogs. Since serum IgE levels have been shown to increase and decrease in parallel to allergen exposure (Masuda et al., 2002; Horiguchi et al., 2008), it will become possible to estimate the exposure levels of each allergen at the tested time point by comparing the serum IgE levels among allergens.

We conclude that the results obtained from the anti-Cry j 1 IgE ELISA were reliable since the dispersion of the results obtained by this assay had low CV (3.1–5.2% and 2.2–8.0% in intra- and inter-assay comparisons, respectively). These CV values are low compared to those in a previous report (Lee et al., 2009), which was considered to demonstrate the reliability of this assay. With such a low inter-assay variance among the results, this ELISA could be an appropriate test to monitor the possible seasonal fluctuation of serum allergen-specific IgE in dogs, since the amounts of

serum allergen-specific IgE in dogs were reported to fluctuate depending on seasonal allergen exposure (Masuda et al., 2002; Horiguchi et al., 2008). By monitoring the fluctuations of IgE to seasonal environmental allergens, it is possible to understand which environmental allergen might be associated with the manifestation of clinical signs in each patient at certain time points. This should aid clinicians to define more accurate therapeutic strategies by determining the specific target allergens at each time point. Another possible application of this ELISA is the evaluation of treatment effect of anti-allergic drugs, allergen avoidance and allergen immunotherapy. Efficacies of the therapies can be evaluated by comparing allergen-specific IgE concentrations before and after treatment (van Halteren et al., 1997; Takagi et al., 2005), which will serve as a valuable tool to evaluate the proof of concept of novel therapies under investigation. The other advantage of a quantitative system is the possible introduction of a standardization system by using pre-quantified reference standard as a universal calibrator. It will be beneficial for veterinarians and pet owners if the test results become able to be compared among different tests and the consistency of the test results are validated by a proper standardization program (DeBoer and Hillier, 2001b). Thus, the quantitative measurement of canine antigen-specific IgE utilizing this ELISA could be beneficial in many aspects of veterinary allergy.

A relationship between the concentration of serum antigen-specific IgE and the onset of *in vivo* allergic reaction has not yet been elucidated in dogs. In general, the test results in antigen-specific IgE ELISA have been compared with those from IDT, since IDT was long considered as a gold standard of the diagnosis of allergy (Mueller et al., 1999; Foster et al., 2003). However, in dogs, since IDT has been shown to exhibit false-positive reactions due to sub-clinical hypersensitivity (Pastorello, 1993; Codner and Tinker, 1995) and false negative results due to the insufficient antigen concentrations that are required to provoke *in vivo* reactions (Hensel et al., 2004), it is difficult to consider IDT as the definitive allergy test in dogs (DeBoer and Hillier, 2001b). Because it is advantageous to measure an absolute quantity of serum allergen-specific IgE by this ELISA, we are currently attempting to evaluate the threshold of IgE concentrations that are associated with *in vivo* allergic reactions instead of comparing the results between ELISA and IDT. In a currently on-going study, we have shown that a concentration of 100–200 ng/ml of antigen-specific IgE in dog serum is sufficient to provoke *in vivo* allergic reaction by Prausnitz–Küstner test using experimental dogs (unpublished data). Similar attempts have been made in human medicine to predict the outcome of food provocation test in human patients with food allergy by defining the threshold of serum allergen-specific IgE titers that elicit allergic reactions (Sampson and Ho, 1997; Sampson, 2001; Komata et al., 2009). However, because the levels of allergen-specific IgE are usually measured in specific units (kU_A/L) in human studies, it is not possible to extrapolate the results to dogs. By further accumulating data in dogs, we consider that this ELISA could be useful to determine a threshold concentration of serum IgE that elicit *in vivo* allergic reaction or clinical symptoms of allergy.

To summarize, we have established a novel ELISA system for the quantitative measurement of allergen-specific IgE in ng/ml in dogs. As we could demonstrate the reliability of the ELISA utilizing CRE-DM, we are aiming at the implementation of CRE-DM in multi-allergen IgE test, which might possibly become a standard allergy test. Since the concept of the quantitative measurement of serum allergen-specific IgE by ELISA utilizing CRE-DM can be further applied not only to dogs but also to other animal species including humans, the ELISA has a potential to be expanded to IgE testing in various species. In the future, comparison of the results on a same scale from medical, veterinary and experimental allergy model studies should enhance the understanding of the pathophysiological mechanism of IgE-mediated allergic diseases.

Conflict of interest statement

This study was funded by Animal Allergy Clinical Laboratories, Inc., Kanagawa, Japan. Dr. Masuda is CEO and stock-holder of this company.

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