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Flow Cytometric Analysis of Lymphocyte Proliferative Responses to Food Allergens in Dogs with Food Allergy

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ABSTRACT. Two different allergy tests, antigen-specific immunoglobulin E quantification (IgE test) and flow cytometric analysis of antigen-specific proliferation of peripheral lymphocytes (lymphocyte proliferation test), were performed to examine differences in allergic reactions to food allergens in dogs with food allergy (FA). Thirteen dogs were diagnosed as FA based on clinical findings and elimination diet trials. Seven dogs clinically diagnosed with canine atopic dermatitis (CAD) were used as a disease control group, and 5 healthy dogs were used as a negative control group. In the FA group, 19 and 33 allergen reactions were identified using the serum IgE test and the lymphocyte proliferation test, respectively. Likewise, in the CAD group, 12 and 6 allergen reactions and in the healthy dogs 3 and 0 allergen reactions were identified by each test, respectively. A significant difference was found between FA and healthy dogs in terms of positive allergen detection by the lymphocyte proliferation test, suggesting that the test can be useful to differentiate FA from healthy dogs but not from CAD. Both tests were repeated in 6 of the dogs with FA after a 1.5- to 5-month elimination diet trial. The IgE concentrations in 9 of 11 of the positive reactions decreased by 20–80%, whereas all the positive reactions in the lymphocyte proliferation test decreased to nearly zero ($P<0.05$), suggesting that lymphocytes against food allergens may be involved in the pathogenesis of canine FA.

KEY WORDS: canine, food allergy, IgE, lymphocytes.

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Adverse reactions to food consist of two different responses: immune-mediated and non-immune-mediated. The disease caused by the former is called food allergy (FA), whereas the latter reaction is recognized simply as food intolerance. FA in dogs causes clinical signs such as chronic dermatitis with pruritus, vomiting, and diarrhea, and it is difficult to diagnose because the clinical signs in skin is similar to those of canine atopic dermatitis (CAD) [21]. To distinguish FA from CAD, the allergens involved must be identified at the onset of clinical signs [4, 10]. An elimination diet trial followed by food provocation has been regarded as the standard for the diagnosis of FA [10]; however, the cumbersome nature of the elimination diet trial makes it difficult to accomplish. Because the trial requires complete cooperation by dog owners for at least 8 weeks to determine whether the clinical signs disappear, a high rate of dropout from the trial has been reported [29]. The difficulty of performing the trial limits the complete assessment and diagnosis of FA in dogs.

The usefulness of testing for immunoglobulin (Ig) E-mediated allergic reactions, or type I hypersensitivity, in the identification of reactive allergens in dogs with FA has been controversial [21]. Intradermal testing examines *in vivo* reactions mediated by the interaction between an allergen and antigen-specific IgE on mast cells in the skin; however, the test has proved unsuccessful for the identification of food allergens in dogs with FA [9, 10, 24]. Serum IgE tests that detect IgE against food allergens have also been found inappropriate because of their low sensitivity in detecting reactive allergens that were identified during food elimination and provocation [9, 10]. Therefore, FA in dogs may be less associated with the pathogenesis of IgE-mediated allergic reactions and instead be caused by other factors.

Different from intradermal testing and the serum IgE test, the measurement of lymphocyte blastogenic response examines an allergic response provoked by a lymphocyte-mediated reaction, known as type IV hypersensitivity. Results from a previous study showed that lymphocyte blastogenic responses to food allergens correlated well with those from food elimination and provocation trials: approximately 82% subject with FA diagnosed after a food elimination and provocation test also tested positive for a lymphocyte blastogenic response using a radioisotope [9]. This result indicates that type IV hypersensitivity against food allergens may be involved in the pathogenesis of FA in dogs. Recently, proliferative T-cells in dogs have been detected with an anti-human CD25 antibody, ACT-1 [14, 15]. Using the antibody, researchers used flow cytometric analysis to detect a culprit food allergen in experimental dogs sensitized with corn [22]; however, there have been no reports to show its usefulness in clinical cases of canine FA.

In this study, subjects were diagnosed with FA using a food elimination and provocation test, and then two types of allergic reactions, type I and type IV hypersensitivity, were examined with a newly established IgE quantification enzyme-linked immunosorbent assay for antigen-specific

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IgE and flow cytometric analysis using ACT-1 for antigen-specific lymphocyte proliferation, respectively.

MATERIALS AND METHODS

Case selection: Dogs with chronic skin diseases were divided into an FA group (13 dogs) or a CAD group (7 dogs) based on criteria previously reported [4]. Animals with parasite infestation with fleas, *Demodex canis*, and scabies were excluded using routine dermatological examinations such as skin scraping and careful observation for flea feces. For further exclusion of scabies infestation, an anti-parasitic drug, either milbemycin oxime or ivermectin, was administered for 2 weeks. If clinical improvement was observed during this treatment, infestation with scabies was confirmed and the subject was excluded. Fungal infection was excluded according to gross observation of skin lesions and ultraviolet light examination. *Malassezia* infection was treated with topically with 2% chlorhexidine or oral itraconazole, 5 mg/kg/day, for at least 2 weeks. Dogs with pyoderma were excluded after a minimum 2-week administration of antibiotics.

Dogs in the FA group showed nonseasonal skin diseases, whereas dogs with CAD displayed skin lesions with seasonal onset. Differential diagnosis of FA and CAD was performed based on food elimination followed by a provocation test with the original diet. The diagnosis with FA was further confirmed by negative intradermal testing results against the environmental allergens tested in this study. The diagnosis was performed in an animal hospital (Fujimura Animal Hospital, Osaka, Japan) owned by one of the authors (M.F.). Five healthy dogs that presented to the hospital for regular vaccination were also included and used as a negative control group (N1 to N5).

Food elimination and provocation test: Food elimination was performed with commercial diets (Prescription Diet z/d ULTRA Allergen-Free Canine, Hill's-Colgate (JAPAN) Ltd., Tokyo, Japan; *Iams Veterinary Formulas Skin & Coat Response*^{TM} *FP/Canine Dry Formula*, Procter & Gamble, Maonson, OH, U.S.A.; SENSITIVITY Control Blue Whiting & Tapioca, Royal Canine Japan, Tokyo, Japan; SENSITIVITY Control Cod & Rice, Royal Canine Japan) or homemade diets (a single ingredient or combination of chicken, pork, sweet potatoes, rice, or potatoes) (Table 1). When the clinical signs were nonseasonal, FA was usually suspected and food elimination was performed using the diets listed above. The optimal diets for food elimination were selected according to the results of the IgE and lymphocyte proliferation tests as well as individual diet history, palatability, and dog owner's preference. Conversely, when clinical signs were seasonal, CAD was suspected, but the food elimination was performed with a low-allergenic commercial diet of either Prescription Diet z/d ULTRA Allergen-free Canine [1, 2] or Aminoprotect Care (Nosan Corporation, Tsukuba, Ibaraki, Japan) [22].

The period of food elimination was 6–8 weeks. When clinical signs resolved (disappearance of pruritus and hair regrowth), food provocation with the original diet was performed for 1 week. If clinical signs recurred during the provocation, the dog was finally diagnosed with FA. The dogs with nonseasonal clinical signs did not respond to food elimination and were diagnosed with CAD.

Intradermal testing: Intradermal testing against environmental allergens including house dust mites; fleas; cotton; cat dander; pollens of ragweed, mugwort, dandelion, Bermuda grass, 7-grass mix (*Poa pratensis*, *Dactylis glomerata*, *Agrostis alba*, *Phleum pratense*, *Anthoxanthum odoratum*, *Festuca elatior*, *Lolium perenne*), and Japanese cedar; *Cladosporium* spp., and *Aspergillus* spp. was performed to support the differentiation of CAD from FA. With the exception of Japanese cedar pollen, the allergen solutions were purchased from GREER (Leinor, NC, U.S.A.) and diluted to the recommended concentrations in phosphate buffered saline (PBS; GREER) according to the manufacturer's instructions. Japanese cedar pollen allergen solution was purchased from Torii Pharmaceutical Co., Ltd. (Tokyo, Japan) and diluted to a concentration of 200 *n*g/m*l* in PBS.

Table 1. Signalments of dogs with food allergy and elimination diets

Dog No.	Breeds	Sex	Age (year)	1st onset (year)	Elimination diets
FA1	French Bulldog	NM	4	2	Homemade (Pork and Corn flakes)
FA2	French Bulldog	SF	2	1	Homemade (Chicken alone)
FA3	Golden Retriever	NM	6	1	Iams® Veterinary Formulas Skin & Coat Response^{TM} FP/Canine Dry Formula
FA4	Great Pyrenees	NM	7	4	Prescription Diet z/d® ULTRA Allergen-free Canine, Pork
FA5	Japanese Terrier	NM	5	1	Homemade (Chicken and Pork)
FA6	Miniature Dachshund	SF	8	3	SENSITIVITY Control Blue Whiting & Tapioca
FA7	Miniature Dachshund	NM	8	1	Homemade (Yogurt, Fermented soybeans, Pork)
FA8	Miniature Pincher	NM	4	2	SENSITIVITY Control Cod & Rice
FA9	Shiba Inu	F	6	3	Homemade (Chicken and Potato)
FA10	Shih Tzu	SF	7	1	Homemade (Pork, Chicken, Salmon, Sweet potato)
FA11	Toy Poodle	NM	2	1	Prescription Diet z/d® ULTRA Allergen-free Canine
FA12	W.H.W. Terrier*	F	6	2	Prescription Diet z/d® ULTRA Allergen-free Canine
FA13	Yorkshire Terrier	SF	5	2	Homemade (Pork, Potato, Rice, Chicken)

F, female; NM, neutered male; SF, spayed female. * West Highland White Terrier.

After the hair of the ventrolateral thorax of the dogs was clipped, 50 μl of the diluted solution of each allergen was intradermally injected with a tuberculin test syringe (Terumo Corporation, Tokyo, Japan). The same volume of histamine solution (GREER) at a concentration of 0.0275 mg/m*l* as a positive control and 0.9% physiological saline solution (GREER) as a negative control were injected in a similar manner. After 15 min, wheal formation was evaluated. When the size of the wheal was equal to or greater than the mean diameter of the wheal between positive and negative controls, the reaction was considered positive.

Antigen-specific IgE quantification (IgE test): The new quantitative antigen-specific IgE test in dogs was carried out using a fluorometric enzyme-linked immunosorbent assay following a previously reported protocol [20], with a modification for the use of 40 allergens in a commercially available test (Animal Allergy Clinical Laboratories, Inc., Sagamihara, Kanagawa, Japan). Because the novel features of the new test enabled us to examine absolute concentrations of antigen-specific IgE in nanograms per milliliter in dog serum [20], we expected to obtain more precise information regarding the relationship between FA and food-allergen-specific IgE in the sera.

The allergens measured in this assay included *Dermatophagoides farinae*; *Dermatophagoides pteronyssinus*; fleas; mosquitoes; cockroaches; pollen of mugwort, ragweed, goldenrod, dandelion, daisy, orchard, sweet vernal, timothy, rye, Bermuda grass, Japanese cedar, birch, and alder; *Aspergillus fumigatus*; *Alternaria alternaria*; *Cladosporium herbarum*; *Penicillium notatum*; beef; pork; chicken; egg white; egg yolk; milk; wheat; soybean; corn; lamb; turkey; duck; salmon; codfish; catfish; capelin; potato; and rice. The allergen solutions (except for Japanese cedar pollen and capelin) were purchased from GREER. Japanese cedar pollen allergen solution was obtained from Torii Pharmaceutical Co., Ltd. Capelin allergen solution was prepared as follows: the fish body was homogenized in PBS, and the supernatant was collected after centrifugation. The allergen solutions were diluted to contain a total protein concentration of 10–20 μg/m*l* and immobilized in 96-well plates. For the standard curve, a combination of rat anti-mouse IgE antibody (R35-72; BD, Franklin Lakes, NJ, U.S.A.) was immobilized in the well plate, and purified mouse IgE (C38–2; BD) with a predetermined concentration was used. To subtract fluorescent units of background reactions, we used pooled normal mouse serum of BALB/c mice for the standard curve and pooled normal dog serum of healthy beagles for the dog samples, both of which were purchased from Zenoaq Nippon Zenyaku Kogyo Co., Ltd. (Fukushima, Japan). The results were calculated according to the standard curve and expressed in nanograms per milliliter. Although the samples were collected from dogs that were sampled on different dates, the IgE test for all samples was performed on the same day.

Sera from 46 healthy dogs were obtained for the negative control samples of the IgE test. The sera were used to determine the cutoff value of serum IgE concentration. Although 40 antigen-specific IgE concentrations could be measured in the healthy dogs, the results of the IgE test against beef-related antigens (beef, milk, and lamb) and fleas were not included in the measurement, because we assumed that beef-related IgE might be increased owing to regular vaccination [19] and past flea infestations.

Lymphocyte proliferation test with food allergens: The lymphocyte proliferation test was carried out when the dogs were present in the same private animal hospital (Fujimura Animal Hospital). Following a previously reported method [22], we cultured peripheral blood mononuclear cells (PBMCs) in RPMI-1640 medium (Sigma, St Louis, MO, U.S.A.) containing 10% fetal bovine serum (Equitech-Bio Inc., Kerrville, TX, U.S.A.) and antibiotics (streptomycin, 100 μg/m*l*, and penicillin, 100 U/m*l*; Sigma) with food antigens including beef, pork, chicken, egg yolk, egg white, milk, corn, soybean, lamb, turkey, duck, salmon, codfish, catfish, capelin, potato, and rice, as purchased or prepared in the IgE test and at a concentration of 5 μg/m*l* at 37ºC under 5% CO_2 in air for 4 days. After human recombinant interleukin-2 (PeproTech Inc., Rocky Hill, NJ, U.S.A.) was added at a final concentration of 5 *n*g/m*l*, the cells were cultured for an additional 3 days. Cells stimulated with concanavalin A (Con A; Sigma) at a concentration of 5 μg/m*l* were used as positive controls for the samples to examine cell proliferation during the culture.

As described in previous reports [14, 15], cultured cells were stained with Alexa 647-labeled anti-canine CD4 antibody (Serotec Ltd., Oxford, U.K.) and phycoerythrin-labeled anti-human CD25 antibody ACT-1 (DAKO, Glostrup, Denmark) to detect proliferating lymphocytes as $CD4^+/CD25^+$ cells. PBMCs for the assay control sample were also stained with isotype-matched antibodies, Alexa647-conjugated rat IgG2a, and phycoerythrin-conjugated mouse IgG1 (Serotec). After dead cell exclusion with 0.015 μg of propidium iodide (BD) in 75–100 μl of PBS, the $CD4^+$ lymphocyte population was analyzed using a FACSCantoII flow cytometer and FACSDiva software (BD).

The percentage of $CD4^+/CD25^{low}$ cells in 2500 $CD4^+$ lymphocytes were further obtained as activated T-lymphocytes in each sample, as it has been reported that the cell population of $CD4^+/CD25^{high}$ cells are regulatory T-lymphocytes [15, 26]. Likewise, a background proliferation of PBMCs in each sample was also obtained without stimulation by food antigens. The final percentages of $CD4^+/CD25^{low}$ cells in $CD4^+$ lymphocytes with food antigen stimulation were calculated after the percentage of background proliferation was subtracted. All experimental procedures were carried out with the same batches of antibodies.

To determine a cutoff value of the percentage of $CD4^+/CD25^{low}$ cells for the lymphocyte proliferation test, PBMCs from 5 healthy beagles (3 females and 2 males between 1 and 3 years of age) kept for experimental purposes (Zenoaq Nippon Zenyaku Kogyo) were treated similarly. The mean percentage + SEM (standard error of mean) was calculated during testing of the food allergens on the beagles. The highest value was used as the cutoff for test results. All

aspects of this study were approved by the Animal Care and Use Committee of Zenoaq Nippon Zenyaku Kogyo.

Changes in responses to food allergens: Repeat IgE and lymphocyte proliferation tests were carried out in 6 of the 13 dogs with FA (FA1, FA4, FA8, FA9, FA10, and FA11) when clinical signs disappeared with food elimination. The serum concentration of IgE and lymphocyte proliferation against the culprit food allergens were compared before and after food elimination.

Statistical analysis: To discern whether the positive results of the IgE and lymphocyte proliferation tests were associated with the clinical diagnosis of disease, we carried out the Fisher exact test (one tailed) at an α level of 0.05 between two groups (FA and CAD; FA and control). The serum IgE concentration and values of the lymphocyte proliferation test were also statistically compared before and after food elimination using the Wilcoxon/Kruskal-Wallis test (two tailed) at an α level of 0.05.

RESULTS

Dogs with food allergy and canine atopic dermatitis: A total of 20 dogs met the criteria for this study. Among them, 13 were diagnosed with FA (FA1 to FA13) and the rest with CAD (CAD1 to CAD7). Dogs with FA also had nonseasonal pruritus, whereas dogs with CAD showed seasonal manifestation of clinical signs, especially in the summer. The dogs with FA showed improvement of clinical signs with food elimination, whereas those with CAD showed no improvement during the food elimination. When the dogs with FA were fed their original diets after food elimination, clinical signs recurred.

The breeds of dogs in this study included French bulldog, golden retriever, Japanese terrier, miniature dachshund, miniature pinscher, Pomeranian, Shiba Inu, shih tzu, toy poodle, West Highland white terrier, and Yorkshire terrier in the FA group (Table 1), and Cavalier King Charles spaniel, Shiba Inu, Maltese, Boston terrier, Welsh corgi, and golden retriever in the CAD group. The age of the dogs in the FA group ranged from 2 to 12 years and included 7 neutered males and 6 females (2 intact and 4 spayed); there was no age or sex predisposition to FA. In the CAD group, age ranged from 2 to 11 years and included 4 males (3 intact and 1 neutered) and 3 females (2 intact and 1 spayed); there was no obvious age or sex predisposition to the disease.

Although the age of first onset of clinical signs in the FA group ranged from 1 to 6 years, 7 (53.8%) of the dogs first showed clinical signs when they were younger than 1 year old. In contrast, in the CAD group, only 2 of the 7 dogs showed clinical signs before 1 year of age.

Intradermal testing: Intradermal testing for environmental allergens was unnecessary to diagnose FA, but it was, with the exception of FA6 and FA13, performed to aid in the diagnosis. With the exception of FA9 and FA12, which showed positive intradermal reaction to house dust mites, dogs with FA had no reactions to the environmental allergens used during intradermal testing. Intradermal testing was carried out in all CAD cases because the test was part of the inclusion criteria for the diagnosis. Of the 7 dogs with CAD, 6 showed positive reaction to house dust mites; CAD2 showed positive reactions against pollens including ragweed, mugwort, dandelion, Bermuda grass, and 7-grass mix.

Serum concentration of antigen-specific IgE in healthy dogs: In most of the serum samples of the 46 healthy dogs, serum concentrations of antigen-specific IgE were not at high levels for 36 of the allergens examined in this study (beef, milk, lamb, and fleas were excluded). Only 4 of the 46 dogs had antigen-specific serum IgE concentrations of 0 *n*g/m*l*; the rest had reactions to 1 to 19 allergens, with antigen-specific serum IgE concentrations ranging from 1 to 1,300 *n*g/m*l*. Because a study of mice indicated that a serum antigen-specific IgE concentration of 100 *n*g/m*l* was enough to induce an *in vivo* immune response in 50% of the mice [27], 100 *n*g/m*l* of serum IgE was chosen as the cutoff value for the IgE test in this study. Of the 46 samples, 29 (63.0%) showed serum antigen-specific IgE levels greater than 100 *n*g/m*l* against one or several allergens; however, among the 36 allergens, the percentage of serum samples with IgE levels greater than 100 *n*g/m*l* was 0–21.7% (potato). Therefore, the IgE test had a specificity of 78.3% when the cutoff value was set at 100 *n*g/m*l*, a specificity comparable to that of the quantitative antigen-specific IgE test in humans [17]. Based on these results, 100 *n*g/m*l* of serum IgE was used as the cutoff value for IgE test results in this study.

Serum concentration of antigen-specific IgE in clinical cases: Among the 13 dogs with FA, only 7 (FA1, FA4, FA6, FA7, FA10, FA11, and FA12) responded to food allergens with serum IgE levels greater than or equal to 100 *n*g/m*l*. IgE levels varied from 102 to 1650 *n*g/m*l* (Table 2). Eighteen allergen reactions were detected, and the food allergens identified with the IgE test were beef, milk, lamb, chicken, turkey, duck, and rice. In the CAD group, 6 out of 7 dogs showed increased IgE against beef, pork, lamb, wheat, corn, capelin, or rice, with levels from 117 to 433 *n*g/m*l*; a total of 12 allergen reactions were detected. In the control group, 2 of the 5 dogs showed increased serum concentrations of IgE against beef, lamb, or potato (see Table 2).

Results of the Fisher exact test showed no significant difference between FA and CAD (P=0.978) or between FA and controls (P=0.500), suggesting that there was no correlation between the clinical diagnosis of these diseases and the identification of food allergens by the IgE test.

Lymphocyte proliferation test against food antigens: The results of the lymphocyte proliferation test in the 5 healthy beagles revealed variation of percentages of proliferative lymphocytes in this study. The percentage of $CD4^+/CD25^{low}$ cells in $CD4^+$ lymphocytes ranged from 0.0 to 0.8% for food allergens and from 8.8 to 29.9% for Con A in the 5 dogs. The mean percentage of the cells against each food allergen in the 5 dogs also ranged from 0.0 to 0.6% with an SEM between 0.0 and 0.7%. When the mean + SEM for all the allergens tested among the 5 dogs was calculated, the highest value was 1.1% lymphocyte proliferation against

Table 2. Food allergens detected with IgE and lymphocyte proliferation tests

Dog No.	Positive allergens in IgE test (*n*g/m*l* of serum IgE concentration)	Positive allergens in lymphocyte proliferation test (% of $CD4^+/CD25^{low}$ cells in $CD4^+$ lymphocytes)
FA1	Beef (1031), Milk (114), Lamb (236)	Egg yolk (3.1), Egg white (8.4), Soy (2.6), Capelin (1.6), Potato (7.9), Salmon (9.1)
FA2	–	Soy (2.7), Duck (2.6), Turkey (2.0)
FA3	–	Pork (1.2), Turkey (3.0)
FA4	Beef (170)	Rice (1.8)
FA5	–	Milk (1.4), Soy (2.1)
FA6	Beef (1345), Milk (305), Lamb (126), Rice (351)	–
FA7	Beef (311), Milk (141)	Pork (2.3), Lamb (1.3), Turkey (1.3)
FA8	–	Milk (2.7), Egg white (1.4), Soy (1.9), Cod fish (1.7)
FA9	–	Wheat (2.8), Egg white (1.7), Soy (1.7)
FA10	Chicken (470), Turkey (120), Duck (522)	Chicken (4.5), Egg white (1.7), Egg yolk (1.5), Milk (1.4), Soy (2.1), Capelin (2.3), Rice (1.9)
FA11	Beef (1650), Milk (226), Lamb (190), Rice (316)	Milk (1.2)
FA12	Beef (291), Rice (102)	–
FA13	–	Wheat (1.9)
CAD1	Beef (295), Capelin (154), Rice (210)	–
CAD2	Pork (167), Wheat (219), Lamb (143)	Egg yolk (2.4)
CAD3	Beef (134)	Egg yolk (1.3)
CAD4	Beef (433), Corn (147), Rice (178)	Chicken (1.4), Wheat (1.6)
CAD5	Beef (117)	Potato (1.6), Rice (1.6)
CAD6	–	–
CAD7	Beef (590)	–
N1	Beef (371), Lamb (162)	–
N2	–	–
N3	–	–
N4	–	–
N5	Potato (152)	–

egg white, which was considered the cutoff value of for the test in this study.

Various food allergens showed positive results on the lymphocyte proliferation test at the levels beyond the cutoff value of 1.1% in dogs with FA (see Table 2). Thirty-three allergen reactions were detected in these dogs. Soy was the most frequently detected allergen (6 of 13 dogs with FA). Egg white and milk were detected in 4 of the dogs. Likewise, turkey (3 dogs), egg white (2), capelin (2), pork (2), rice (2), wheat (2), potato (1), salmon (1), duck (1), chicken (1), lamb (1), and codfish (1) were detected. The largest lymphocyte proliferation response in the positive allergens was 9.1% against salmon in FA1. Among the increased percentages of lymphocyte proliferation test, the mean and SEM were 2.6 and 2.0%, respectively. Con A-stimulated samples showed increased lymphocyte proliferation with a mean percentage of 22.9% and SEM of 11.3%, which were regarded as a positive control that confirmed cell proliferation in the blood samples during the culture.

In dogs with CAD, 5 food allergens, including egg yolk (2 dogs), chicken (1), wheat (1), potato (1), and rice (1), were detected using the lymphocyte proliferation test. The percentage of lymphocyte proliferation in dogs with CAD varied from 1.3 to 2.4, with a mean of 1.7 and an SEM of 0.33. No food allergens were detected in the 5 healthy dogs (see Table 2).

Overall 11 of the 13 dogs with FA tested positive for at least one food allergen in the lymphocyte proliferation test. Likewise, 4 of 7 dogs with CAD and 0 of 5 healthy dogs were positive. The Fisher exact test showed no significant difference between FA and CAD (P=0.207), suggesting that there was no association between the clinical diagnosis of those diseases and the identification of food allergens using the lymphocyte proliferation test; however, there was a significant difference between the FA and control groups (P<0.05), suggesting that the identification of food allergens by the lymphocyte proliferation test was associated with the differentiation of the dogs with FA from the healthy dogs.

Change in allergic reactions against offending food antigens: In 6 clinical cases of FA (FA1, FA4, FA8, FA9, FA10, and FA11) the IgE and lymphocyte proliferation tests were repeated after the clinical signs disappeared during an elimination diet (Table 3 and Table 4). The duration of the elimination diet varied from 1.5 to 4.5 months (see Table 3). The IgE test was repeated for the 11 food allergens that raised serum IgE levels above 100 *n*g/m*l* before food elimination in 4 of the 6 dogs (see Table 3). Serum concentration of IgE against these food allergens decreased after food elimination (except for milk and lamb in FA1) in a pre- and post-food elimination ratio of serum IgE concentration of 0.2 to 0.8 (reduction rate of 20–80%). The IgE concentrations for milk and lamb in FA1 increased in a pre/post ratio of 1.6 and 1.1, respectively. There was no significant difference in IgE concentration before and after food elimination (P=0.200). Among the 9 food allergens against which IgE concentration decreased post-food elimination, only 2 (turkey in

Table 3. Change in serum immunoglobulin E concentrations between before and after food elimination

Dog No.	Elimination period (months)	Offending food allergens	IgE (*n*g/m*l*)		IgE ratio
			pre	post	post/pre
FA1	5	Beef	1,013	787	0.8
		Milk	114	188	1.6
		Lamb	236	248	1.1
FA4	4.5	Beef	170	140	0.8
FA8	1.5	None			
FA9	3	None			
FA10	1.5	Chicken	470	252	0.5
		Turkey	120	65	0.5
		Duck	522	253	0.5
FA11	1.5	Beef	1650	412	0.2
		Milk	226	113	0.5
		Lamb	190	78	0.4
		Rice	316	147	0.5

Dogs FA8 and FA9 displayed no positive food allergens detected by the IgE test.

Table 4. Change in lymphocyte proliferation responses between before and after food elimination

Dog No.	Offending food allergens	Lymphocyte proliferation test (% of $CD4^+/CD25^{low}$ cells in $CD4^+$ lymphocytes)	
		pre	post
FA1	Capelin	1.6	0.0
	Potato	7.9	0.6
	Egg yolk	3.1	0.0
	Egg white	8.4	0.0
	Soy	2.6	0.0
	Salmon	9.1	0.0
FA4	Rice	1.8	0.1
FA8	Egg white	1.4	0.2
	Milk	2.7	0.3
	Soy	1.9	0.7
	Cod fish	1.7	0.3
FA9	Wheat	2.8	0.0
	Egg white	1.7	0.5
	Soy	1.7	0.2
FA10	Chicken	4.5	0.0
	Egg white	1.7	0.0
	Egg yolk	1.5	0.0
	Milk	1.4	0.0
	Soy	2.1	0.0
	Capelin	2.3	0.1
	Rice	1.9	0.0
FA11	Milk	1.2	0.0

FA10 and lamb in FA11) resulted in IgE concentrations lower than 100 *n*g/m*l*, whereas IgE concentrations for the rest of the food allergens remained above 100 *n*g/m*l*.

The lymphocyte proliferation test was repeated for 22 kinds of offending food allergens in the 6 dogs (see Table 4). All of these values decreased after the elimination diet. Although most decreased to 0.0 %, some (potato in FA1; egg white, milk, soy, and codfish in FA8; egg white and soy in FA9; and capelin in FA10) remained between 0.1 and 0.7%. There was a significant difference in the results of the lymphocyte proliferation test before and after food elimination ($P<0.05$).

DISCUSSION

In the13 clinical cases with FA in this study, there was no predisposition of any breed to FA; however, most of the breeds were small or medium. Although a predisposition of certain dog breeds to FA has not yet been confirmed, the French bulldog and shih tzu are candidates for predisposition because they have been identified in another study on FA in dogs in Japan [9]. A larger number of clinical cases should be evaluated to confirm predisposed breeds.

There was no age and sex predisposition to FA in dogs in this study. The first onset of clinical signs occurred earlier than 1 year of age in 53.8% of the dogs with FA in this study, however, which supports the data in previous studies. One study conducted in Japan showed that 46% of dogs with FA began showing clinical signs between 1 and 3 years of age [9]. Studies in other countries have reported first onset of clinical signs earlier than 1 year of age in 37.5 % [3] and 46.5% [7] of dogs with FA. These observations suggest that FA might be acquired at an early age in dogs and that exposure to food allergens during the post-weaning period may be one of the important factors that induce FA.

Various offending food allergens were identified using both the IgE test and the lymphocyte proliferation test in dogs with FA in this study. When compared with those in dogs with CAD, the number of food allergens that tested

positive in the lymphocyte proliferation testing in dogs with FA was greater than that detected with the IgE test. This result indicates that the lymphocyte proliferation response to food allergens might be more helpful than IgE testing for identifying offending food allergens. A comparable tendency was reported in a previous study using dogs with FA, in which 82% of offending food allergens were detected with a test that measured lymphocyte blastogenic responses using a radioisotope, whereas the IgE test was useful for only 31% of the allergens [9].

In the FA group in our study, 6 dogs displayed no food allergens during IgE testing, whereas only 2 dogs were negative for food allergens when lymphocyte proliferation testing was performed (see Table 2). Statistical analysis revealed an association between the clinical diagnosis of FA and positive response to food allergens in the lymphocyte proliferation test, not in the IgE test, when dogs with FA were compared to healthy controls. Thus, the pathogenesis of FA in dogs might be associated more with lymphocyte-mediated reactions than with IgE-mediated reactions. In humans with FA, it has also been reported that measuring lymphocyte proliferative responses against food allergens was useful for detecting offending food allergens [11]. Taken together, we consider the lymphocyte proliferation test to be useful for understanding the pathogenesis of FA in dogs and helpful in its diagnosis.

The usefulness of the IgE test for identifying offending food allergens might be limited in dogs because of the possible increase of IgE against beef-related components that results owing to regular vaccination. In this study, we found no relationship between the clinical diagnosis of FA and the identification of food allergens using the IgE test. It has been reported that IgE production against beef components occurred in dogs that received vaccination because the vaccine products can contain fetal bovine serum [18, 19]. In this study, an increase in beef-specific IgE was detected in 5 of the 13 dogs with FA, 5 of the 7 dogs with CAD, and 1 of the 5 healthy dogs, and no other food allergens were detected with such high frequency. This relatively high frequency of beef-specific IgE detection might also be associated with vaccination, as previously reported [18, 19]. Because beef-specific IgE can be found in dogs receiving vaccination, it is difficult to diagnose beef allergy with the IgE test alone. The development of future tests to distinguish beef-specific IgE elevated by vaccination from that elevated by beef allergy is important.

In this study, the number of offending food allergens identified by the IgE test or the lymphocyte proliferation test varied from 1 to 7 among the dogs in the FA group, suggesting that dogs could be sensitized to various food allergens. When dogs acquire an allergic reaction against foods, two factors should be considered. First, it is important to know what food ingredients the dogs had been fed when acquiring allergic reaction. In mice, allergic reaction tended to be acquired before weaning [25]. If dogs are fed food allergens before weaning, it is presumable that the risk for developing FA would be enhanced. The difference in the number of offending food allergens among the dogs with FA may reflect the difference in the number of food allergens they consumed during the infant period.

Second, the difference of the offending food allergens might be due to genetic difference in dogs. Allergen components are recognized by T-cells through T-cell epitopes, which are restricted by the interaction between T-cell receptors and major histocompatibility complex II (MHC II) [16]. If the genetic backgrounds of T-cell receptor and MHC II are similar between the dogs, then allergen recognition in the dogs should also be similar. Because a large difference in T-cell epitopes has been reported in both experimental dogs and dogs in clinical settings that were sensitized to Japanese cedar pollen [13], a large variation in the genes encoding T-cell receptor and MHC II may be present among dogs. Such genetic variation can also cause differences in offending food allergens in dogs.

The food allergens detected by the IgE and lymphocyte proliferation tests were not always same in this study. This variation may be due to difference in the types of allergic reactions. Type IV hypersensitivity is delayed hypersensitivity caused by the cell-mediated reactions of antigen-specific T-cells independent of type I hypersensitivity caused by IgE, and both types of allergic reaction are known to exist together in human patients clinically diagnosed with atopic dermatitis [6, 28]. It remains unknown which type of allergic reaction occurs in each food allergen at sensitization. Serum IgE was mostly induced when intragastric acid suppression was carried out in mice orally sensitized to ovalbumin [5]. It is possible that allergenicity of food components in the gastrointestinal tract may be a factor in determining whether type I or type IV hypersensitivity is acquired.

The serum IgE concentration cutoff value might be a matter of debate because a different cutoff value might change the number of food allergens identified by the IgE test. A serum antigen-specific IgE concentration of 100 *n*g/m*l* was considered a suitable cutoff value to indicate the possible manifestation of clinical symptoms. This value was determined based on the findings of a mouse study [27]. The cut-off value of antigen-specific IgE in dogs must be confirmed by further study that focuses on the specificity and sensitivity of the IgE test. In allergic reactions, because the value eliciting clinical symptoms presumably differs from dog to dog and from allergen to allergen, a cut-off range rather than a single cut-off point might be more appropriate.

The IgE and lymphocyte proliferation tests were repeated in 6 FA cases in which clinical signs disappeared after an elimination diet. For the 11 food allergens tested, IgE concentrations against 9 decreased after the elimination diet, although the IgE concentrations were still above the cutoff value except for turkey in FA10 and lamb in FA11. Since there was no significant statistical difference between IgE levels before and after food elimination in this study, IgE might not be appropriate to evaluate effects of elimination diets in 1.5–4 months of food elimination. Considering that serum IgE decreased but were still present without antigen challenge for several months, IgE-producing plasma cells

might have been alive for that long period of time in the bone marrow or spleen of the dogs and continuing to produce IgE in the peripheral blood.

The serum half-life of IgE has been reported to be only 12 hr in mice [30] and 1 to 5 days in humans [23]. Plasma cells in bone marrow are responsible for the maintenance of serum IgE titers, and their survival time has been determined to be 3 months in mice [12]. This information suggests that the life expectancy of IgE-producing plasma cells in dogs would also be several months. Because plasma cells in bone marrow do not proliferate [12], there should be no increase in serum IgE unless new plasma cells from memory B cells are added into bone marrow. If it becomes possible to block memory B cells from developing into plasma cells, IgE production can be eliminated, and every IgE-mediated allergic reaction could be controlled. Because therapeutic strategies that aim to eliminate plasma cells are being developed [31], therapies that eliminate both memory B cells and IgE-producing plasma cells would also be desirable.

The values that were positive for food allergens during lymphocyte proliferation testing decreased to negative ranges after food elimination in this study. This phenomenon suggests that the duration of food elimination allowed sufficient time for food-allergen-reactive lymphocytes to disappear from the peripheral blood. Memory T-cells for these offending food allergens might still exist in central immune organs such as spleen and lymph nodes, however. In human patients with Japanese cedar pollinosis, the clone size of pollen-specific T-cells decreased but their memory function was maintained for 8 months after the pollen season [8]. Because the time required to induce natural cell death of memory lymphocytes is unknown, it is difficult to determine when dogs could resume eating their original diets.

In summary, reactions to offending food allergens in dogs with FA were examined with two new allergy testing methods: the IgE test and the lymphocyte proliferation test. Although the combination of these tests was useful for identifying offending food allergens and diagnosing FA in dogs, the lymphocyte proliferation test detected more food allergens than did the IgE test. Both tests were considered to be useful in the monitoring of allergic reactions during food elimination.

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