

## Lymphocyte Blastogenic Responses to Food Antigens in Cats Showing Clinical Symptoms of Food Hypersensitivity

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**ABSTRACT.** Three cats were diagnosed as having food hypersensitivity by food elimination and oral food provocation tests. Twelve allergenic food ingredients were identified by oral food provocation test in the 3 cats. Of the 12 food ingredients, 9 offending food antigens were shown to be positive in a lymphocyte stimulation test; however, none of them were positive in antigen-specific IgE testing, and only four food antigens were positive in intradermal testing. The stimulation indices in the lymphocyte stimulation tests for the 9 food ingredients were found to be decreased after the cats were fed elimination diets. The present study demonstrates that the lymphocyte stimulation test reflects an immunologic reaction involved in food hypersensitivity and can help identify allergenic food ingredients in feline food hypersensitivity.

**KEY WORDS:** feline, food hypersensitivity, lymphocyte stimulation test.

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Adverse food reactions are considered to consist of food hypersensitivity and food intolerance. The pathogenesis of food hypersensitivity is not well understood in cats, and we principally depend on inference from studies in human food hypersensitivity. Food hypersensitivity in cats shows various clinical signs, such as pruritic skin diseases and gastrointestinal signs [7]. It has been estimated that food hypersensitivity accounts for as many as 1% of all feline dermatoses in general practice. No age and sex predilections have been documented [22].

Diagnosis of food hypersensitivity is difficult in cats [15, 22]. An oral food provocation test following a food elimination test is recognized as the most reliable diagnostic procedure in cats. Novel protein [12], hydrolyzed protein [4, 20], and homemade diets are generally used as elimination diets; however, many cats are not willing to eat elimination diets. In such cases, it is difficult to carry out a food elimination test.

Serum antigen-specific IgE testing is considered to be unreliable for identification of offending food allergens in cats with food hypersensitivity [6]. In cats with food hypersensitivity showing gastrointestinal signs, the rate of agreement for positive food allergens between IgE testing and an oral food provocation test was shown to be only 28% [6]. On the other hand, in cats with food hypersensitivity showing dermatological signs, the sensitivity of

serum antigen-specific IgE testing has not been reported. Although intradermal testing is used as a method for identification of allergens in cats with atopic dermatitis, its diagnostic value in feline food hypersensitivity has not yet been reported. In humans, intradermal testing is considered to be unreliable for identification of food allergens in food hypersensitivity because of its low sensitivity and specificity [5]. Similarly, the sensitivity of intradermal testing in dogs with food hypersensitivity is also known to be low [9].

On the other hand, the lymphocyte stimulation test has been shown to be useful for the diagnosis of food hypersensitivity in humans [10] and dogs [8]. There have been no reports on the lymphocyte stimulation test in feline food hypersensitivity, although a cat with Japanese cedar pollinosis was shown to have an antigen-specific lymphocyte blastogenic response against its pollen antigen [13]. In this report, the lymphocyte stimulation test was used to identify offending food allergens in cats.

Three cats with possible food hypersensitivity were referred to the Veterinary Medical Center of the University of Tokyo. All 3 cats showed aggravation of clinical signs possibly related to the ingestion of specific food ingredients. A food elimination test and oral food provocation test were carried out according to the procedures reported previously [3, 22]. As an elimination diet, an optimal diet was selected depending on the clinical history in each cat. When apparent improvement of clinical signs was recognized by the owners after food elimination for 3 to 8 weeks, the cats were then challenged with several food ingredients for 7 days. The food ingredients for the provocation test were selected based on the dietary history and the willingness of the owners to feed them to their cats in each case. Food ingredients included in the foods that had been fed

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before referral were selected for the food provocation tests.

Serum concentrations of antigen-specific IgE were measured by ELISA using recombinant human Fc $\epsilon$ R1 $\alpha$  (ALLERCEPT Detection System, Heska Veterinary Diagnostic Laboratories, Fort Collins, CO, U.S.A.) [21]. Eighteen foods (cooked beef, cooked chicken, cooked pork, cooked fish mix [cod, halibut, tuna], whole egg, milk, cooked rice, wheat grain, cooked corn, soybean, potato, cooked lamb, brewer's yeast, barley, whey, rabbit, turkey and venison) were available.

Intradermal testing was performed for 9 food allergens (beef, chicken, cow's milk, hen's egg, rice, wheat, corn, cod and tuna) as described previously [13]. The irritant threshold for food allergens was not determined in cats; therefore, the concentrations of 9 food allergens were set at 1000 PNU as reported in dogs previously [14]. These allergen extracts were purchased from a commercial laboratory (GREER Laboratories, Lenoir, NC, U.S.A.). Histamine solution (0.0275 mg/ml) and 0.9% saline solution were used as the positive and negative controls, respectively. Before performing intradermal testing, administration of corticosteroids and antihistamines was discontinued for more than one week in all the cats. After the cats were sedated by intramuscular injection of medetomidine hydrochloride (Domitor, Orion Corporation, Espoo, Finland) (0.04 mg/kg) and midazolam (Dormicum, Yamanouchi, Tokyo, Japan) (0.3 mg/kg), 0.05 ml of each allergen solution was intradermally injected by one clinician into the clipped skin of the ventrolateral thorax using a skin test syringe with a 26-gauge needle. The diameters of wheals were measured 10 to 15 min after the injection. When a reactive wheal was palpable and erythematous, and the size was at least greater than that of the positive control, it was considered to be a positive reaction to the injected allergen.

The lymphocyte stimulation test was performed at 2 different time points. For the first point (provocation phase), blood samples were collected within 3 weeks after the positive reaction disappeared in the food provocation test. For the second point (elimination phase), blood samples were collected when the cats showed no clinical symptoms of food hypersensitivity at more than 3 weeks after the provocation phase. Heparinized peripheral blood samples were diluted with an equal volume of phosphate buffered saline and layered on Ficoll-Hypaque (Nycomed Pharma AS, Oslo, Norway). After centrifugation at 350 g for 40 min at room temperature, a layer of peripheral blood mononuclear cells (PBMCs) fraction was obtained and suspended in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, U.S.A.) containing 10% heat-inactivated fetal calf serum (Intergen Co., Purchase, NY, U.S.A.) and antibiotics (100 U of penicillin and 0.1 mg of streptomycin per ml) at a cell count of  $1.25 \times 10^6$ /ml. A cell suspension of 200  $\mu$ l was allocated to each well of a 96-well plate and incubated at 37°C for 48 hr under stimulation with each of the food extracts (GREER Laboratories) (5  $\mu$ g/ml) that were used in the oral food provocation tests. Blastogenic responses of the antigen-stimulated and unstimulated PBMCs were measured with the assay by incorporation of  $^3$ H-thymidine (38 kBq/

well) for an additional 18 hr. Incorporation of  $^3$ H-thymidine was measured by a liquid scintillation counter (LSC-5100, Aloka, Tokyo, Japan). The stimulation index was calculated with the following formula: stimulation index=(cpm of cultures stimulated with food antigens)/(cpm of unstimulated cultures). A stimulation index equal to or greater than 2.0 was recognized as a positive reaction based on criteria used for the lymphocyte proliferation test in humans [11]. As a control group, 5 clinically healthy domestic short hair cats (3 males, 2 females) were used. They were 10 to 11 months old (mean, 10.4 months old). These cats were subjected to food provocation challenges with 9 food ingredients (beef, chicken, cow's milk, hen's egg, rice, wheat, corn, cod and tuna) and showed no clinical signs after intake of these foods. In these cats, the lymphocyte stimulation test was performed 1 week after the oral provocation tests.

Case 1 was a 7-year-old, castrated, male, Abyssinian cat with pruritus that first occurred at the age of 1 year. The cat's skin lesions were characterized as nonseasonal erythema with crusting of the lower jaw, lips, eyelids and inguen. The dermatological symptoms partially responded to glucocorticoid therapy; however, they relapsed after termination of the therapy. Routine dermatological examinations were performed in order to exclude other pruritic skin disorders, such as infections with microorganisms (bacterial and mycotic infections), flea infestation, scabies and demodicosis. Based on the clinical symptoms, history and dermatological examinations, this cat was considered to have food hypersensitivity. In this case, the clinical signs improved after feeding with an elimination diet (Hill's Prescription Diet Feline z/d Low Allergen, Hill's Pet Nutrition, Topeka, KS, U.S.A.) for 4 weeks. After apparent improvement of the clinical signs by the food elimination test, the cat was subjected to provocative challenges with 6 kinds of food ingredients: beef, cow's milk, hen's egg, rice, corn and tuna. Erythema and pruritus in the eyelids and inguen reappeared after provocation challenges with beef, cow's milk, hen's egg, corn and tuna but not with rice (Table 1). Corn and tuna were found to induce erythema with pruritus approximately 24 hr after provocation. As for the other three kinds of foods, pruritus occurred approximately 2 hr after provocation.

Case 2 was an 8-year-old, castrated, male, domestic short-haired cat, showing nonseasonal pruritus and erythema in the skin of the face and inguen since the age of 3 years. These skin lesions improved after glucocorticoid therapy; however, they relapsed several months after cessation of the therapy. Food hypersensitivity was also suspected after ruling out infectious dermatological disorders by routine dermatological examinations. Although the complete blood cell count and serum chemistry profile did not show any abnormalities, the cat was found to be positive for feline immunodeficiency virus (FIV) antibody (Snap FeLV/FIV Combo Test, IDEXX, Westbrook, ME, U.S.A.). This cat was considered to be an asymptomatic carrier of FIV. For the food elimination test, this cat was fed an elimination diet (Hill's Prescription Diet Feline z/d Low Allergen, Hill's Pet Nutrition), and complete resolution of the pruritus

Table 1. Results of an oral provocation test, lymphocyte stimulation test, antigen-specific IgE test and intradermal test in 3 cats with food hypersensitivity

Cat No.	Food ingredients	Oral food provocation test	Lymphocyte stimulation test		Antigen-specific IgE test	Intradermal test
			Provocation phase	Elimination phase		
1	Beef	+	<b>7.1</b>	0.9	-	+
	Cow's Milk	+	<b>5.8</b>	1.1	-	-
	Hen's Egg	+	<b>4.8</b>	0.8	-	-
	Corn	+	<b>2.3</b>	1.2	-	+
	Tuna	+	<b>2.6</b>	1.2	-	-
	Rice	-	1.8	NT	-	-
2	Chicken	+	<b>2.3</b>	0.9	-	-
	Hen's Egg	+	<b>5.0</b>	0.9	-	-
	Cow's Milk	+	1.0	0.8	-	-
	Cod	+	0.9	1.3	-	-
	Tuna	+	1.0	1.0	-	-
	Beef	-	1.2	NT	-	-
	Rice	-	1.0	NT	-	-
3	Cod	+	<b>2.9</b>	1.0	-	+
	Tuna	+	<b>2.7</b>	0.9	-	+

+, Positive reaction; -, negative reaction; NT, not tested. Stimulation indices more than 2.0 (categorized into positive results) are shown in bold.

was observed. This cat was then subjected to provocative challenges with beef, chicken, cow's milk, hen's egg, rice, cod and tuna. Pruritus became apparent 1 to 2 hr after oral food provocations with chicken, cow's milk, hen's egg, cod and tuna but not with beef and rice.

Case 3 was a 6-year-old, spayed, female, domestic short-haired cat with a long history of nonseasonal intermittent vomiting and diarrhea since the age of 8 months. The cat owner reported that the clinical signs became worse after feeding tuna, resulting in vomiting at least 3–4 times per day. However, the cat did not lose its appetite, and its general condition was fairly good even when it was vomiting. The results of fecal examination, abdominal radiography and abdominal ultrasound did not show any abnormal findings. On blood examination, the cat was positive for FIV antibody but was considered to be an asymptomatic carrier. Based on these findings, we suspected that the cat had food hypersensitivity against tuna. For the elimination diet, Hill's Prescription Diet Feline d/d Lamb and Rice (Hill's Pet Nutrition) was chosen. Cod and tuna were selected for the oral food provocation test. This cat showed vomiting 2 days after initiation of the oral food provocation tests with cod and tuna. The clinical signs of this case resolved immediately after refeeding the elimination diet.

The results of antigen-specific IgE testing in the 3 cats with food hypersensitivity are shown in Table 1. Although the 3 cats examined in this study showed a recurrence of symptoms after feeding with 2–5 food ingredients, they did not show any positive results for serum IgE directed to the offending food antigens. In Case 3, positive reactions to 4 food antigens (whole egg, cooked rice, cooked corn and brewer's yeast) were found, but oral food provocation tests for them were not carried out. No positive result was

obtained in the antigen-specific IgE test against the 18 food ingredients in the 5 control cats.

The results of the intradermal testing against food antigens are shown in Table 1. Case 1 showed positive reactions to beef and corn but negative reactions to cow's milk, hen's egg, cod and rice. No reactive wheals were observed after injection with food allergens in Case 2. In Case 3, positive reactions to cod and tuna were found. Positive wheals were also detected against beef, cow's milk, chicken, hen's egg and corn; however, oral provocation tests for these 5 food ingredients were not performed.

In the 3 cats diagnosed with food hypersensitivity, the lymphocyte blastogenic responses were examined after stimulation with the food extracts listed in Table 1. In Case 1, 5 kinds of food ingredients (beef, cow's milk, hen's egg, corn and tuna) were positive in the lymphocyte stimulation test (stimulation indices, 2.3–7.1). The stimulation index for rice was less than 2.0 in Case 1, and this cat did not show relapse after feeding rice. In Case 2, the lymphocyte stimulation test was performed for 7 food allergens (chicken, hen's egg, cow's milk, cod, tuna, beef and rice). Positive blastogenic responses were found by stimulation with extracts of chicken and hen's egg; however, the results for cow's milk, cod, tuna, beef and rice were negative. The stimulation indices for the 5 offending food allergens (chicken, hen's egg, cow's milk, cod and tuna) varied from 0.9 to 5.0. Two non-offending food allergens (beef and rice) did not show proliferative responses. In Case 3, positive reactions were found by stimulation with cod and tuna extracts, with stimulation indices of 2.9 and 2.7, respectively. In all 5 control cats, the lymphocyte stimulation test was performed for 9 food allergens: beef, chicken, cow's milk, hen's egg, rice, wheat, corn, cod and tuna. The stimulation indices for all 9 food

ingredients were less than 2.0 (range, 0.5–1.4).

The results of the lymphocyte stimulation test in the 3 cats were compared between the provocation and elimination phases (Table 1). In Cases 1 and 3, the stimulation indices for the offending food antigens were all positive in the provocation phase, but they decreased to less than 2.0 in the elimination phase. In Case 2, the stimulation indices for chicken and hen's egg also decreased to levels less than 2.0 in the elimination phase. The stimulation indices for cow's milk, cod and tuna were less than 2.0 in both the provocation and elimination phases in this case.

In the 3 cats with food hypersensitivity examined in this study, 9 of the 12 offending food allergens could be identified with the lymphocyte stimulation test. In the elimination phase, the stimulation indices for the offending food allergens in the 3 cats decreased more than 3 weeks after feeding with the elimination diet. The positive results of the lymphocyte stimulation test would indicate the presence of cellular immune response mechanisms in the pathogenesis of food hypersensitivity, as shown in humans [2]. In feline food hypersensitivity, immunologic parameters have not been available, except for the serum food antigen-specific IgE directed to food ingredients [6]. Thus, the present study would provide an immunologic test in feline food hypersensitivity. In addition, the decline in stimulation indices after feeding elimination diets observed in the present study has also been reported in human patients with food hypersensitivity [1]. It is conceivable that the number and reactivity of lymphocytes responsive to food antigen increases in the provocation phase and decreases in the elimination phase. Therefore, the lymphocyte stimulation test may be one of the benchmarks to monitor the allergic response in cats with food hypersensitivity.

In the 3 cases, 9 (75.0%) of the 12 offending food allergens could be identified by the lymphocyte stimulation test. Of the 12 offending food ingredients, none was positive in the antigen-specific IgE testing, and only 4 (33.3%) were positive in the intradermal testing. Therefore, agreement with the results of the food provocation test was considered to be higher for the lymphocyte stimulation test than for the antigen-specific IgE testing and intradermal testing. We previously reported a similar study on canine food hypersensitivity [8]. In humans, a similar study was also reported in non-immediate type food hypersensitivity [10]. Consequently, performance of a lymphocyte stimulation test for possible food allergens may be useful in predicting offending food allergens in cats with food hypersensitivity. Since the number of cats with food hypersensitivity examined in this study was small, further study of a large number of feline cases is needed to assess the diagnostic value of the lymphocyte stimulation test.

Although the lymphocyte stimulation test may be useful in detecting offending food allergens in feline food hypersensitivity, there remain at least 3 limitations. Firstly, we evaluated the sensitivity of the lymphocyte stimulation test; however, its specificity could not be precisely assessed in this study. Because we performed only 3 lymphocyte stimulation tests for non-offending food allergens in Cases

1 and 3, the number of subjects was not enough to estimate the specificity. Next, blastogenic responses were seen in the provocation phase, but the lymphocyte stimulation test was not performed at their initial presentations. If lymphocyte blastogenic responses to food allergens are detected before the food elimination test, the lymphocyte stimulation test would be useful as a screening test to identify the offending food allergens. Lastly, the cut-off value of the stimulation index could not be defined with certainty for the lymphocyte stimulation tests for feline food hypersensitivity. In this study, we employed the cut-off value (2.0) used in humans with food hypersensitivity [11], but it may not always be appropriate for feline food hypersensitivity. In the 5 healthy control cats used in this study, the stimulation indices for 9 food allergens were 0.5 to 1.4 (mean  $\pm$  standard deviation [SD],  $1.0 \pm 0.3$ ). Mean plus 2 SD was calculated to be 1.6; therefore, the cut-off value used in humans (2.0) was considered to be tentatively appropriate in feline food hypersensitivity. For determination of the cut-off value of the stimulation index in cats, further study to test a large number of healthy cats will be needed.

In this study, the results of the lymphocyte stimulation test showed some future possibilities. The lymphocyte stimulation test may be useful in identifying allergenic components of food. In cow's milk hypersensitivity in humans, the lymphocyte stimulation test has been used to identify the major allergenic components in cow's milk [17]; therefore, the system could be applied in order to identify the allergenic components in feline food hypersensitivity. Moreover, T cell epitopes of the major allergens would also be clarified by the blastogenic responses of the lymphocytes by cultivation with a set of overlapping peptides of the allergen molecules [19].

In addition, the lymphocyte stimulation test may provide a clue to understanding the immunologic reactions in feline food hypersensitivity. By analyzing the cell surface markers, the characteristics of the proliferated lymphocyte after stimulation with offending food allergens will be identified. Moreover, measurement of the cytokine profile in culture supernatants will be important to examination of the function of proliferated lymphocytes and the subsets of helper T cells. For example, IL-4, IL-13 [18] and TNF- $\alpha$  [16] were detected in the culture supernatants of a lymphocyte proliferation assay for cow's milk hypersensitivity in humans. Therefore, a similar study may be helpful to elucidate the mechanism of food hypersensitivity in cats.

In conclusion, increased blastogenic responses to food allergens were found in 3 cats with food hypersensitivity, indicating the presence of circulating lymphocytes reactive to food antigens in the peripheral blood in these cases. Therefore, positive responses in a lymphocyte stimulation test mean that immunologic reactions are found in cats with food hypersensitivity. The lymphocyte stimulation test may provide a laboratory procedure that could be used in diagnostic and immunologic studies for feline food hypersensitivity.

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