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# Hydrolyzed diets may stimulate food-reactive lymphocytes in dogs

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**ABSTRACT.** Hydrolyzed proteins are often prescribed for dogs with food hypersensitivity in food elimination programs. However, the potential of these diets to stimulate lymphocyte-mediated hypersensitivity is currently unknown. In this study, two commercially available hydrolyzed diets for dogs, D-1 (Aminopeptide Formula Dry, Royal Canin Japon, Tokyo, Japan), and D-2 (Canine z/d Ultra Dry, Hill's-Colgate (Japan) Ltd., Tokyo, Japan), were analyzed to identify residual proteins or peptides, as well as activated helper T-lymphocyte reactions in dogs with suspected food hypersensitivity. Proteins and peptides with molecular weights >1 kDa (majority 1.5–3.5 kDa) were detected in both diet extracts with sodium dodecyl sulfate polyacrylamide gel electrophoresis, and size exclusion chromatography. When peripheral blood mononuclear cells (PBMC's) from 316 dogs with suspected food allergies were cultured with hydrolyzed diet extracts, flow cytometry analysis revealed detectable levels of $CD25^{low}$ helper T-lymphocytes stimulated by D-1 and D-2 in 91 of 316, (28.8%), and 75 of 316 (23.7%) samples, respectively. These data indicated that the extracts contained proteins or peptides large enough to activate the lymphocytes. The percentages of $CD25^{low}$ helper T-lymphocytes stimulated by D-1 and D-2 extracts increased to 38.7% and 29.6%, respectively, in 186 of the original 316 samples (186/316, 58.9%), also reactive to poultry-related antigens. Thus, both poultry-related antigens, and D-1 and D-2 diet extracts may activate helper T-lymphocytes. These results demonstrate that hydrolyzed diets may contain proteins that stimulate helper T-lymphocytes, and may not be effective for treating all dogs with food hypersensitivity.

**KEY WORDS:** diet, dog, food allergy, hydrolyzation, lymphocyte

Elimination diets often contain low molecular weight (MW) proteins and peptides resulting from enzymatic digestion (hydrolysis) of proteins from source materials [2, 10, 20]. Hydrolyzed protein diets are considered therapeutic for companion animals since they may prevent allergic reactions due to food hypersensitivity [2]. In humans, IgE may recognize protein allergens with MW ranging from 5–50 kDa, therefore, hydrolyzed proteins in elimination diets must have MW lower than 5 kDa to prevent allergic reactions [29]. A recent study in dogs demonstrated that a canine diet made from hydrolyzed poultry-feather meal containing 95% hydrolyzed proteins with MW ≤1 kDa did not induce clinical reactions in any of the dogs allergic to chicken. However, another diet containing 78% hydrolyzed chicken liver proteins with MW ≤1 kDa did induce allergic reactions in 40% of the dogs [2].

A clinical study of dogs diagnosed with food hypersensitivity after food restriction and provocation found type IV hypersensitivity lymphocyte-mediated reactions in 82% of the dogs, however, type I hypersensitivity was rarely detected [8]. Another report evaluating clinical cases of dogs with chronic dermatitis and suspected food hypersensitivity confirmed this trend [5]. In addition, a study conducted on dogs with gastrointestinal symptoms clinically diagnosed as inflammatory bowel disease, found peripheral lymphocytes reactive to at least one food antigen in all the animals [12]. Flow cytometry analysis revealed that the type IV lymphocytes associated with hypersensitivity were $CD4^+$ helper T-lymphocytes [5, 21]. Theoretically, these types of lymphocytes are reactive to proteins or peptides that do not induce IgE-mediated reactions [10, 14]. Therefore, it is clear that clinical use of hydrolyzed diets, as part of food elimination programs, may not be appropriate for all dogs with food hypersensitivity.

Small peptides (T cell epitopes) recognized by helper T-lymphocytes are displayed by MHC (Major Histocompatibility Complex) class II molecules found on antigen-presenting cells, such as macrophages, dendritic cells, and B-lymphocytes, which engulf antigens via phagocytosis. In previous studies, Wu *et al.*, 2002, and Masuda *et al.* 2004, experimentally determined the amino acid (AA) sequences of common allergens by mapping with overlapping peptides containing *ca.* 15 AA's covering all the

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protein allergen sequences [17, 30]. The estimated MW of the 15 AA length peptides ranged from 1–3 kDa [6, 17, 23], depending on AA composition. In a 2007 study, Kabuki and Joh showed that a peptide MW range of 1–3 kDa was critical for induction of a T-lymphocyte reaction in a human infant with casein-reactive T-lymphocytes. The study results demonstrated that helper T-lymphocytes were stimulated by mildly hydrolyzed casein with MW of ≥2 kDa, but nothighly hydrolyzed casein with MW ≤1 kDa [10]. Moreover, in another study, a specific T-lymphocyte helper clone could recognize a peptide consisting of only 5 AA, with a MW <1 kDa [7]. These results suggest that helper T-lymphocytes may recognize hydrolyzed proteins in a clinical setting, therefore, lower hydrolyzed-protein MW are desirable to prevent reactions.

Since T-cell epitopes are linear peptides, allergen AA sequences may be conserved in similar species, and therefore cause allergic reactions due to T-lymphocyte cross-reactivity. In a recent study, the major allergens from Japanese cedar and cypress Cry j 1 and Cha o 1, contained AA sequences with approximately 80% homology [26], and also contained identical T-cell epitope sequences recognized by human T-lymphocytes reactive to both Cry j 1 and Cha o 1 [23]. The Cry j 1 and Cha o 1 T-cell epitopes were also identical to various tree pollen and vegetable species epitopes [9, 31]. Alpha-parvalbumin, a major allergen from chicken meat, contains AA sequences that are highly conserved (greater than 80% homology) in poultry and livestock animals, and in a previous study resulted in induction of cross-reactive human IgE [15]. In 2018, Bexley *et al.* reported that dog serum IgE cross-reactivity between chicken and fish was related to nine proteins common to both species [1]. Alpha-actin, a highly conserved gene in vertebrates, was identified as one of the nine proteins [3]. Consequently, since T-lymphocytes may be widely cross-reactive to low MW allergens from taxonomically similar species, it is possible that T-lymphocytes could react to proteins or peptides even after hydrolysis.

In this study, the MW and antigenicity of protein extracts from two types of hydrolyzed diets, Aminopeptide Formula Dry (Royal Canin Japon) and Canine z/d Ultra Dry (Hill's-Colgate (Japan) Ltd.), were examined to determine whether hydrolyzed proteins could stimulate helper T-lymphocytes in dogs with suspected food hypersensitivity.

## MATERIALS AND METHODS

### *Protein extraction from hydrolyzed diets*

Two types of commercially available hydrolyzed diets (D-1, Aminopeptide Formula Dry, Royal Canin Japon, known as Anallergenic, Royal Canin, Aimargues, France and D-2, Hill's Prescription Diet, Canine z/d Ultra Dry, Hill's-Colgate (Japan) Ltd.) were purchased. When the study was initiated, three product lots of D-1, and two lots of D-2 were available. The protein extraction protocol was similar to the one previously reported [11]. Briefly, one gram of crushed kibbles from each diet was minced and suspended in 10 m*l* of physiological saline, and subsequently ground with glass beads under shaking conditions at a speed of 6,000 rpm for 1 min, in an ULTRA-TURRAX® Tube Drive (IKA® Werke GmbH & Co., KG., Staufen, Germany). This procedure was repeated three times with one-min intervals. The suspension was maintained at 4°C for 30 min to remove sediments after homogenization, and the supernatant was collected and centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was filtered and sterilized after centrifugation, with a 0.45 $\mu$m pore size PES membrane, and protein concentrations were measured with ultraviolet (UV) light absorbance at a wavelength of 280 nm. Finally, the extracts were analyzed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and size-exclusion chromatography, and tested for lymphocyte stimulation.

### *SDS-PAGE*

The hydrolyzed diet and chicken meat extracts (Greer Laboratories, Inc., Lenoir, NC, USA) were separated by SDS-PAGE in a 5–20% gradient polyacrylamide gel (ATTO Corp., Tokyo, Japan) using a previously described method [11]. The total protein amounts in D-1, D-2, and chicken meat extract were adjusted to 10 $\mu$g per lane. After electrophoresis, the gel was stained with Coomassie brilliant blue, (CBB) (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan), or silver stain reagent (Cosmo Bio Co., Ltd., Tokyo, Japan) for protein band visualization.

### *Size-exclusion chromatography*

Size-exclusion chromatography of the extracts was performed using slight modifications of a previously reported procedure [11]. A Superdex 200 10/300 GL column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was equilibrated with phosphate buffered saline (PBS). Each sample was eluted isocratically at a flow rate of 0.5 m*l*/min in PBS, and protein elution was monitored with UV absorbance at 280 nm. The column was calibrated with a Gel Filtration Low MW Kit (GE Healthcare Bio-Sciences AB), containing different MW protein standards: ferritin (MW 440 kDa), aldolase (MW 158 kDa), conalbumin (MW 75 kDa), ovalbumin (MW 43 kDa), carbonic anhydrase (MW 29 kDa), ribonuclease A (MW 13.7 kDa), and aprotinin (MW 6.5 kDa).

### *Lymphocyte stimulation*

As previously reported [5, 12, 25], lymphocyte proliferation of PBMC's isolated (by veterinarians in private animal hospitals) from dogs with suspected food hypersensitivity, was investigated in a routine assay with 18 types of food antigens in a commercial laboratory (Animal Allergy Clinical Laboratories, Inc., Sagamihara, Japan). The food allergens for the standard lymphocyte assay were extracted from beef, pork, chicken, egg white, egg yolk, milk, wheat, soybean, corn, lamb, turkey, duck, salmon, cod fish, catfish, potato, and rice, and all were prepared and purchased from Greer Laboratories Inc. as previously described [5, 12, 25]. However, capelin was prepared by homogenization of the dried fish body in PBS. Detailed clinical symptoms and dog history were unknown, however, dermatological and/or gastrointestinal symptoms were suspected. Additionally, no information on the status of allergen exposure and treatment history was available for the dogs providing the blood samples. The blood samples were kept at

4°C and shipped to the laboratory by refrigerated courier services within 1–3 days after collection from local veterinarians. Briefly, PBMC's were purified from whole blood with Ficoll-Hypaque (Lymphoprep, Axis-shield, Oslo, Norway) under centrifugation. The cell viability was determined by exclusion of trypan blue (Sigma-Aldrich, Inc., St. Louis, MO, USA). When more than 95% of cells were viable, the PBMC's were cultured for seven days in RPMI-1640 medium (FUJIFILM Wako Pure Chemical Corp.), containing 10% fetal bovine serum, 100 $\mu$g/m*l* streptomycin, and 100 U/m*l* penicillin (Sigma-Aldrich), at 1 $\mu$g/m*l* antigen concentration for each allergen. Following the standard assay with 18 types of food allergens, the remaining PBMC's were also tested in a similar manner with extracts from D-1 and D-2. On Day 4 of the culture, recombinant human IL-2 (Pepro Tech Inc., Rocky Hill, NJ, USA) was added at a concentration of 100 U/m*l*. Subsequently, the cultured PBMC's were stained with antibodies, and analyzed via flow cytometry.

*Flow cytometry*

As previously described [5, 14], the antibodies used in the flow cytometry analysis included anti-dog CD4 rat antibody labeled with Alexa Fluor 647 (Clone YKIX302.9) (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and anti-human CD25 antibody labeled with phycoerythrin (Clone ACT-1) (DAKO Cytomation, Glostrup, Denmark), as well as isotype controls such as Alexa Fluor 647-labeled purified rat IgG2a (Bio-Rad Laboratories), and PE-labeled mouse IgG1 (Bio-Rad Laboratories). Stained dead cells were excluded by propidium iodide incorporation. Finally, $CD4^+CD25^+$ cells were measured in a lymphocyte gate with FACS Canto II and FACS Diva software (Becton Dickinson, San Jose, CA, USA). We determined the number of activated helper T-lymphocytes against each antigen by calculating the percentage of $CD4^+$ $CD25^{low}$ lymphocytes in 2500 $CD4^+$ lymphocytes [18, 19]. We defined activation as ≥0.4% CD4-positive T-lymphocytes, since at least ten positive cells (0.4% of 2,500 cells) confirmed the presence of a distinct cell population.

When considering clinical use of hydrolyzed diets in a food elimination program, induction of a lymphocyte response in dogs allergic to poultry-related antigens should be a major concern, since the diets examined in this study contained poultry-related proteins, such as feather meal or chicken meat. In this study, the positive lymphocyte (≥0.4%) response to poultry-related antigens was compared to the response induced with hydrolyzed proteins. When a positive lymphocyte response to two or more poultry-related antigens was detected in a sample, the maximum antigen value was designated as representative of a lymphocyte response. Positive lymphocyte responses to poultry-related antigens and hydrolyzed diets were statistically analyzed with Spearman's rank correlation coefficient. In addition, since positive lymphocyte responses might not always be detected, qualitative comparisons were also conducted. The $\chi^2$ test was utilized to analyze categorical data from samples with positive (≥0.4%) and negative (<0.4%) lymphocyte responses, and from poultry-related antigens and hydrolyzed diets. Statistical analyses were conducted with XLSTAT software (Addinsoft, Paris, France).

A value of 1.2% has been considered a threshold of clinical manifestation in dogs with food hypersensitivity [5]. Therefore, we counted the number of samples with hydrolyzed diet response values of ≥1.2%, to calculate a lymphocyte response potentially associated with clinical manifestation.

## RESULTS

*Hydrolyzed diet protein molecular weights*

SDS PAGE gels of the D-2 extract processed with CBB and silver stain revealed multiple broad bands with MW from 3.5–24 kDa, and faint bands at approximately 24 kDa and 52 kDa. No bands were detected in the D-1 extract processed with CBB, and silver staining revealed only one faint band with a MW slightly larger than 24 kDa (Fig. 1). Since the protein amounts in all lanes (D-1, D-2 and chicken meat) were adjusted to 10 $\mu$g, the differences in visualized bands were due to protein component differences in the samples.

Size-exclusion chromatography revealed 6–8 protein waveform peaks with different molecular weights in both D-1 and D-2 (Figs. 2 and 3). Based on the standard protein reagents, the two major peaks had an estimated MW >1 kDa, approximately 1.5 kDa and 3.5 kDa. A peak with a MW >440 kDa (the largest standard protein) was detected in the D-2 extracts. All three D-1 product lots, and two D-2 product lots displayed similar wave patterns (Fig. 3). Size-exclusion chromatography data obtained in a different laboratory (Toray Research Center, Inc., Tokyo, Japan), revealed waveform peaks similar to our results (data not shown).

*Lymphocyte responses to hydrolyzed proteins and poultry-related antigens*

Of 316 PBMC total samples examined in the study, positive lymphocyte responses (≥0.4%) against D-1 and D-2 extracts were detected in 91 (91/316, 28.8%), and 75 (75/316, 23.7%) samples, respectively (Table 1).

We applied Spearman's rank correlation coefficient (due to lack of normality) to determine if there was a correlation between positive lymphocyte responses in the D-1 or D-2 extracts, and the maximum response with poultry-related antigens. However, no correlations were observed, (D-1, rs=0.256; D-2, rs=0.239). Positive lymphocyte responses (≥0.4%) were observed with at least one poultry-related antigen, such as chicken, duck, turkey, or chicken egg, in 186 of 316 total samples (186/316, 58.9%). The D-1 extracts elicited positive lymphocyte responses in 72 samples (72/186, 38.7%), and the D-2 extracts elicited responses in 55 (55/186, 29.6%) samples (Table 1). D-1 and D-2 extracts elicited positive responses in 130 samples that did not exhibit positive lymphocyte responses to any poultry-related antigens; 20 samples (20/130, 15.4%) responded to D-1, and 19 samples (19/130, 14.6%) to D-2. Application of the Chi-squared test demonstrated that there was no association between the maximum percentage of positive lymphocyte responses to poultry-related antigens, and the positive responses to the D-1 or D-2 extract.

Only seven (7/316, 2.2%) D-1 samples and six D-2 samples (6/316, 1.9%) elicited lymphocyte reactions with values ≥1.2%.

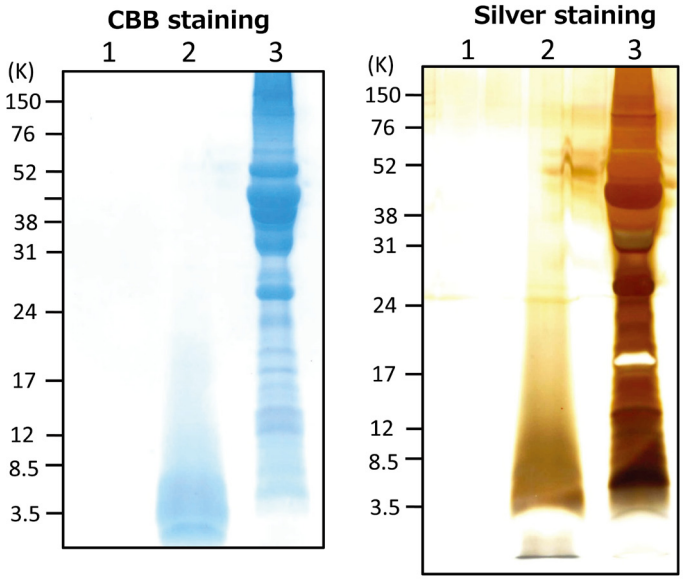


**Fig. 1.** SDS-PAGE of D-1 (Lane 1), D-2 (Lane 2), and chicken meat (Lane 3) extracts with Coomassie brilliant blue (CBB) and silver staining. The total protein amount applied to each lane was 10 $\mu$g. Note that there were no visualized bands in Lane 1. SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

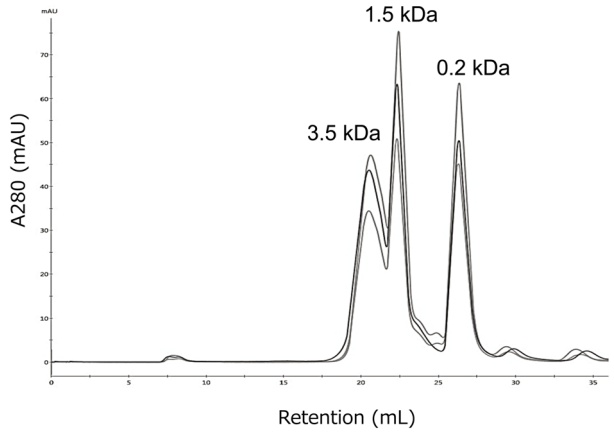


**Fig. 2.** Size-exclusion chromatography of D-1 extracts. The assay was repeated with three different product lots. Similar wave patterns were detected in all product lots.

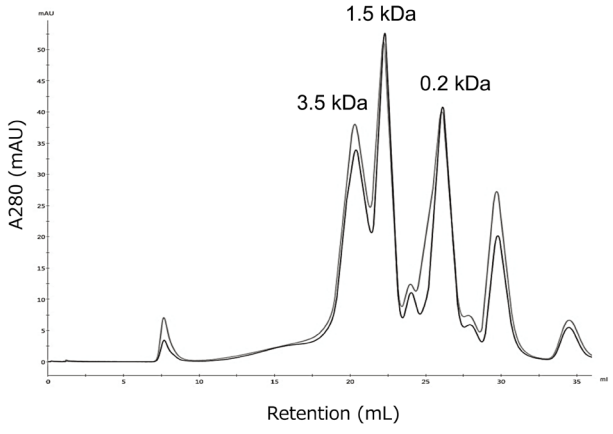


**Fig. 3.** Size-exclusion chromatography of D-2 extracts. The assay was repeated with two different product lots. Similar wave patterns were detected in all product lots.

## DISCUSSION

Hydrolyzed diets are considered effective for food elimination programs in companion animals with food hypersensitivity, since they contain proteins or peptides with molecular weights that are typically too low to stimulate allergic reactions. However, clinical cases of dogs with food hypersensitivity that did not respond positively to food elimination programs with hydrolyzed diets have been reported [2]. In addition, a previous report revealed that several carbohydrate proteins with molecular weights ranging from 21–67 kDa, and those recognized by serum IgE from individual cases of dogs with suspected food hypersensitivity, were detected in the same commercial diets examined in this study [22]. In this study, SDS-PAGE gels of D-1 and D-2 extracts revealed bands with slightly higher MW than 24 kDa. In addition, a peak with an extremely high MW (>440 kDa) was detected only in D-2 extracts with size exclusion chromatography. These results suggest that type I hypersensitivity may occur in clinical cases where serum IgE is reactive to the proteins.

Previous reports showed that a type IV hypersensitivity allergic reaction mediated by T-lymphocytes recognizing small peptides

**Table 1.** In each sample, the presence or absence of helper T-lymphocytes reactive to hydrolyzed foods (D-1 and D-2) and poultry proteins was compared. Samples were stratified at 0.4% of reactive helper T-lymphocytes, since it was considered in this study that 0.4% was the minimum percentage to show reactive helper T-lymphocytes

| | Poultry | | Total |
|---|---|---|---|
| | ≥0.4%[a)] | <0.4%[b)] | |
| D-1 ≥0.4% | 72 | 19 | 91 |
| D-1 <0.4% | 114 | 111 | 225 |
| Total | 186 | 130 | 316 |
| D-2 ≥0.4% | 55 | 20 | 75 |
| D-2 <0.4% | 131 | 110 | 241 |
| Total | 186 | 130 | 316 |

a) When helper T-lymphocyte response to any of chicken, turkey, duck, egg white, and egg yolk was 0.4% or more, the sample was counted as ≥0.4%. b) When helper T-lymphocyte response to any of chicken, turkey, duck, egg white, and egg yolk was less than 0.4%, the sample was counted as 0.4% <.

not recognized by IgE, was observed in dogs with food hypersensitivity [5, 8]. In this study, the lower MW proteins and peptides in the hydrolyzed diet extracts were tested for induction of lymphocyte-mediated allergic reactions. Size-exclusion chromatography of D-1 and D-2 extracts revealed proteins and peptides approximately 1.5 kDa and 3.5 kDa MW (Fig. 2). Previous studies suggested that proteins or peptides of this size were large enough to induce helper T-lymphocyte reactions, since synthetic peptides between *ca.* 1–1.5 kDa MW were used in experiments to detect helper T-lymphocyte reactions to T-cell epitopes [13, 18]. In addition, flow cytometry results in this study demonstrated a lymphocyte response to low MW proteins and peptides in the hydrolyzed diets. These results confirm that even low MW proteins and peptides may not be small enough to avoid a T-lymphocyte response in dogs with food hypersensitivity. Consequently, although hydrolyzed diets have been clinically effective in food elimination programs for many dogs with food hypersensitivity as previously reported [2], it is possible that T-lymphocyte allergic reactions might be prompted by residual proteins in hydrolyzed diets, and therefore these diets should not be considered appropriate for all dogs with food hypersensitivity.

Although potential induction of T-lymphocyte reactions against hydrolyzed protein diets has been suspected, induction of clinical symptoms related to food hypersensitivity in dogs was considered unlikely for three reasons. First, in this study, T-lymphocyte responses with values ≥1.2% were detected in only seven of 316 (2.2%), and six of 316 (1.9%) cases stimulated by D-1 and D-2, respectively. Since 1.2% has been considered the threshold for clinical manifestation of canine food hypersensitivity [5], hydrolyzed diets should be safe in most cases. Second, in dogs fed hydrolyzed diets, gastrointestinal digestion should decrease the molecular size of proteins or peptides, possibly preventing antigenicity against T-lymphocytes. For example, frequent gastrointestinal digestion of collagen *in vitro* results in small peptides with MW <1 kDa [24], a molecular size consistent with common T-cell epitopes [17, 23]. Therefore, any T-cell epitopes remaining in hydrolyzed protein diets should be further reduced in size during gastrointestinal digestion. Third, since the protein sources in the hydrolyzed diets examined in this study were poultry-feather meal (D-1), and chicken meat (D-2) [2], they may have included T-cell epitopes taxonomically similar to many poultry proteins. In general, T-cell epitopes are conserved among major allergens with taxonomic similarity [28], and cross-reactivity against multiple allergens can occur [9, 23, 31]. T-cell epitopes should be conserved in poultry-related allergens; this may explain the "bird-egg syndrome" phenomenon of human allergic reactions to some avian meats [13] and feathers [27]. However, in this study, we found no significant correlation between positive lymphocyte responses against D-1 or D-2 extracts, and poultry-related allergens, suggesting that cross-reactivity of T-lymphocyte reactions may not be very common. Although further study on T-cell epitopes of poultry-related antigens in dogs is necessary, at present, hydrolyzed diets are considered to have a low risk for T-lymphocyte cross-reactivity. Consequently, a food elimination clinical program using hydrolyzed diets should be successful in most dogs with food hypersensitivity.

We detected one faint but clear protein band slightly larger than 24 kDa MW in silver stained SDS-PAGE gels of D-1 and D-2 extracts. This was inconsistent with results from a previous report where wide ranges of residual proteins from 21–67 kDa MW were found in the same hydrolyzed diets examined in this study [22]. Possibly, this may be due to differences in protein extraction procedures. The previous report described a homogenization procedure with a tissue homogenizer, *versus* the easy-to-use tabletop homogenizer utilized in the present study. In addition, protein extraction efficiency may be increased when the starting material is pulverized rather than homogenized [16], therefore an optimized method for protein extraction from hydrolyzed diets should be developed.

In this study, protein concentrations were measured with UV light absorption at 280 nm because dye-staining methods, such as the Bradford method, were unsuccessful for the D-1 extracts (data not shown). This result was consistent with the SDS-PAGE results since CBB staining did not visualize any D-1 extract proteins in the lower MW ranges (3.5–24 kDa), whereas broad protein bands were visualized with both CBB and silver stain in the D-2 extracts. It is likely that proteins were not stained in the D-1 extracts due to structural damage of AA. CBB reacts with many types of AA, including aromatic and basic, as well as with the

AA N-terminus [4], likely destroyed in the D-1 extract. In comparison, during silver staining, silver ions bind to AA sulfhydryl and hydroxyl groups, subsequently visualized with paraformaldehyde in a basic pH solution that reduces silver ions to metallic silver [17]. Notably, the D-1 extract proteins were also not visualized with silver stain, suggesting that the AA sulfhydryl and/or hydroxyl groups might have been damaged in the extraction procedure. In addition, regardless of the negative staining results, the D-1 extract protein concentrations were easily measured with UV light, which is absorbed by AA benzene rings. These results suggest that AA conformation in the D-1 extracts might have been partially damaged, but not destroyed. However, since proteins in the D-2 extracts were detected after both CBB and silver staining; the protein extraction procedure could not have affected protein conformation in the D-1 extracts. Therefore, proteins in the D-1 source material, *i.e.* hydrolyzed feather meal, should be analyzed to determine whether hydrolyzation could potentially cause changes in protein conformation.

Since the lymphocyte response is influenced by several factors in cell culture conditions such as reactive antigen or peptide concentrations, duration of antigen stimulation, cell concentrations, and ratios of reactive lymphocytes and antigen-presenting cells, T-lymphocyte reactions would not necessarily always be detected in this study. False-negative results might occur depending on cell culture conditions; although a lymphocyte response ≥1.2% was designated as the food hypersensitivity detection threshold in dogs [5, 9]. Therefore, the significance of this study was the *in vitro* detection of lymphocyte responses in dogs stimulated by hydrolyzed diet extracts, previously reported in humans [10], but not in dogs. Lymphocyte responses would be controllable in constant culture conditions, and comparable to lymphocyte responses stimulated by different antigens in other studies. These results could be obtained in dogs, (as in studies of human helper T-lymphocyte proliferation), by determination of the specific co-culture ratio between antigen-presenting cells, and CD4 positive lymphocytes, autologously isolated from blood samples [1, 3].

The PBMC's utilized in this study were sent by veterinarians for a previously described standard lymphocyte proliferation assay [5, 12, 25], and after the assay was completed, the remaining cells were used to examine T-lymphocyte responses to the hydrolyzed diets. Therefore, we did not have access to the history and clinical features of the dogs and blood samples. Ideally, before blood samples are submitted for lymphocyte proliferation assays, dogs with suspected allergies should be clinically diagnosed with food hypersensitivity based on their responses to food elimination programs and provocation [8]. Typically, only a limited number of dogs may be accepted for this type of analysis since the diagnostic procedure is complex, and may not always be successfully completed. In this study, we examined 316 samples, and although the sample inclusion criteria were not strictly defined, the large sample size permitted a comprehensive analysis of T-lymphocyte responses to hydrolyzed diets in dogs with potential food hypersensitivity. However, further studies should be conducted to confirm T-lymphocyte reactions to hydrolyzed diets in dogs with food hypersensitivity.

In summary, the current study demonstrated that hydrolyzed diets contain proteins and peptides with sufficiently high molecular weights to stimulate T-lymphocytes. Therefore, it is important to avoid hydrolyzed diets in food elimination programs when treating dogs previously diagnosed with lymphocytes reactive to poultry-related antigens.

CONFLICT OF INTEREST. K. Masuda is the Chief Executive Officer and a stockholder of Animal Allergy Clinical Laboratories, Inc.

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