Evaluation of the immunogenicity of dietary proteins in cats and the influence of the canning process

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**Objective**—To characterize the antigen-specific immune response to dietary proteins in cats and evaluate whether there was a qualitative or quantitative difference between the responses to dietary proteins when those proteins were fed unprocessed or as part of a canned diet.

**Animals**—14 healthy domestic shorthair cats.

**Procedure**—Cats were fed 2 dietary proteins (soy and casein) either as unprocessed aqueous suspensions or as part of canned diets for 21 days. Serum IgG and IgA and salivary IgA were assayed by indirect ELISA, and antigen-specific proliferation of mesenteric lymph node-derived lymphocytes was determined.

**Results**—Robust serum IgG and IgA responses to dietary proteins were elicited, irrespective of the form in which they were fed. Salivary IgA responses to unprocessed proteins were not detected. However, a significant salivary IgA response to the protein isolated from the canned casein diet was observed in cats fed canned casein but not in those fed unprocessed casein. Lymphocyte proliferation to the antigens was slight, and there were no significant differences between groups.

**Conclusions and Clinical Relevance**—Results indicated that cats develop robust serum IgG and IgA responses to dietary proteins when fed as either aqueous suspensions or as part of canned diets. For certain proteins, there may be an increase and a qualitative difference in the immunogenicity of canned diets, compared with unprocessed proteins. Canned diets may not be ideal for management of cats with enteritis. (Am J Vet Res 2004;65:1427–1433)

Adverse reactions to food are common in cats and are reportedly present in as many as 29% of all cats with chronic gastrointestinal disease. In addition, inflammatory bowel disease is the single most common cause of chronic gastrointestinal disease in cats, and novel antigen and hydrolyzed protein diets are reportedly effective in management of this disease. However, although the involvement of immunologic mechanisms in a proportion of these adverse reactions is suspected, it has not been proven. The normal immunologic response to ingested dietary antigens in cats has not yet been described.

As obligate carnivores, cats have evolved on a highly digestible diet. In keeping with this is the short intestinal tract of the cat, which suggests that cats may not be suited to poorly digestible diets. Commercial canning processes decrease protein digestibility, and this has biologically important effects in cats.

In rodents and rabbits, intact particulate and insoluble antigens are preferentially absorbed across the intestine through M cells overlying the Peyer's patches. Classically, such antigens tend to invoke active immunity appropriate for microorganisms. In contrast, soluble antigens have been found to be associated with oral tolerance. Oral tolerance can be abrogated when soluble proteins are fed in oil-in-water emulsions, resulting in robust systemic humoral responses. This effect may also have relevance to the pet-food industry, in which interactions between dietary proteins and lipids in canned or extruded diets during cooking and manufacturing processes could feasibly cause novel interactions that would not be present in the proteins' and lipids' native states.

In stark contrast to rodents is the intestinal response in chickens, in which particulate antigens induce tolerance, whereas soluble antigens provoke active immunity. If the physical nature of the proteins within the natural diet of a species dictates how the intestinal immune system has evolved, this may have special relevance to species that are commonly fed diets different from their ancestors. Such may be the situation for cats, although to the authors' knowledge, results of studies specifically evaluating oral tolerance in cats have not yet been published.

Commercial pet foods are subjected to substantial heating during the manufacturing process. Heat treatment changes the 3-dimensional conformation of proteins. Although this may disrupt some antigens, it may equally uncover previously hidden antigenic determinants or create new ones. The Maillard reactions also occur at high temperatures and involve reactions between certain amino acids and reducing sugars that generate less digestible compounds called melanoids, which give a characteristic brown color. Melanoids tend to be less digestible and less soluble, and certain melanoids may be more allergenic than the original uncooked protein.

The purposes of the study reported here were to characterize aspects of the antigen-specific immune
response to dietary proteins in cats and to evaluate whether there was a qualitative or quantitative difference between the response to dietary proteins when those proteins were fed unprocessed or as part of a canned diet. We hypothesized that the immunogenicity of dietary protein would be increased as the result of the commercial canning process, compared with the same protein when fed unprocessed to naive cats.

Materials and Methods

Animals and husbandry—All cats were from a specific pathogen-free colony and were 7 to 9 months old at the time of study. In addition, cats were chosen from queens that had been fed a commercial diet that did not contain any source of the trial proteins during pregnancy and lactation, and the study cats were fed the same standard diet from weaning and throughout the study period. In addition, none of the cats had been vaccinated or exposed orally or parenterally to the proteins used in the study or to wheat or ovalbumin antigens. This was to ensure that the cats were naive to the trial proteins and to known cross-reactive proteins to prevent the induction of oral tolerance caused by prior exposure. Cats were housed individually in the Nutrition and Pet Care Center in a constant temperature environment with a 12-hour light-and-dark cycle. Water was available to cats ad libitum. Maintenance of the cats was in accordance with the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act, and the study was approved by the University of California, Davis, Animal Use and Care Committee.

Exclusion criteria included any history of vomiting or diarrhea; any evidence of fecal parasites from 3 fecal flotations, performed via zinc sulphate centrifugation, within 1 month of the beginning of the study; pruritic skin disease; or systemic illness as determined by results of physical examination, CBC, and serum biochemical analyses.

Diets—Two canned diets were prepared. One contained a soy isolate and the other diet contained casein as the protein source. The raw ingredients were mixed with water and heated to 80° to 82°C with stirring until gelatinization of the starch occurred. The ingredients were then pumped through a colloid mill followed by homogenization at 2,000 psi and a 3-hour heat-up period and a 10-minute cooling at the end of sterilization. The canned diets were complete and balanced (Table 1). The caloric proportions were 23% protein, 49% fat, and 28% carbohydrate, and the solids content was 21%. The dietary concentrations of protein, amino acids, choline, taurine, and vitamins and minerals in both diets met or exceeded the requirements as defined by the National Research Council. The macronutrient proportions were chosen to provide a concentration of protein consistent with, and to mimic the physicochemical conditions of, certain commercially available diets. They were also selected on the basis that the total protein intake (canned diet plus purified protein) each day would not notably exceed that which would be considered reasonable. The total daily protein intake (canned plus unprocessed aqueous suspension) represented 48% on a dry matter basis (DMB). Purified powdered soy and casein proteins were used for the unprocessed protein feeds and were the same mass as the protein fed in the canned form. This was administered as a suspension in water. On this basis, each cat received 2 g of each protein/kg of body weight daily. Cats received the same caloric content relative to their maintenance energy requirements (MERs) as calculated from the formula:

\[
MER = 70 \times \left(\text{body weight in kg}\right)^{0.75}
\]

Study design—Cats were randomized into 2 groups of 7 cats; each group contained 4 males and 3 females. Two weeks before the start of the study, percutaneous gastrostomy feeding tubes were placed into each cat during general anesthesia. On each day from day 0 to day 28 at 8 AM and 2 PM, cats were fed equal amounts of the canned diet, and at 8 PM, they were fed a bolus of unprocessed protein as an aqueous suspension. One group was fed the canned soy diet, followed by an aqueous suspension of unprocessed casein protein; the other group was fed the canned casein diet, followed by an aqueous suspension of unprocessed soy protein. Of their total daily caloric intake, 50% was supplied via the canned diet and protein suspension and 50% was via the standard weaning diet, which was available ad libitum overnight. Blood was collected for serum, and saliva was collected on days 0, 7, 14, and 28. Biopsy specimens of the mesenteric lymph nodes were obtained via a keyhole midline incision during general anesthesia on days 7 and 21.

Test antigens—The 4 antigens used in both ELISA and cell culture experiments included unprocessed casein, unprocessed soy, protein isolate from the canned soy diet, and protein isolate from the canned casein diet. Protein isolates obtained from the canned diets were prepared via an adaptation of the method of Pastorello and Trambaioli. Briefly, samples of the diets were mixed in a weight-to-volume ratio of 1:4 in 0.1 mol/L of PBS solution (PBSS; pH, 7.4) and stirred for 24 hours at 4°C. This was followed by ammonium sulphate precipitation and centrifugation, dialysis for 24 hours against 1mM of 2-mercaptoethanol in distilled water by use of a 2.000-d cut off dialysis membrane in a sample-to-dialysate ratio of 1:100 and a dialysate change at 12 hours. The dialysate was paper-filtered and lyophilized. Prior to use in ELISA, the reconstituted suspension was filtered through a 0.22-µm sterile centrifugal filter. The protein concentration following reconstitution was assayed by use of the biuret method and adjusted accordingly. Samples of the canned protein isolates were tested by use of a commercial service for the presence of endotoxin to assess their suitability for use in cell culture with the Limulus amebocyte lysate gel-clot test. Endotoxin concentrations ranged from 7.3 to

Table 1—Composition of canned diets fed to cats.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Casein diet (g/kg of dry matter)</th>
<th>Soy diet (g/kg of dry matter)</th>
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</thead>
<tbody>
<tr>
<td>Casein</td>
<td>250</td>
<td>0</td>
</tr>
<tr>
<td>Soy isolate</td>
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<td>300</td>
</tr>
<tr>
<td>Corn oil</td>
<td>265</td>
<td>265</td>
</tr>
<tr>
<td>Corn starch</td>
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<td>Glucose</td>
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<tr>
<td>Vitamin supplement</td>
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<td>10</td>
</tr>
<tr>
<td>Choline citrate</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Taurine</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Supplemental arginine*</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Supplemental methionine*</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Arachis oil (essential fatty acids)</td>
<td>0.5</td>
<td>0.5</td>
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</tbody>
</table>

*The arginine and methionine contents of the diets were adjusted to ensure each diet exceeded the National Research Council nutrient requirements for cats.

For all individual amino acids, there was < 10% difference of mean daily intake between cats fed canned casein and those fed canned soy diets.
were incubated in a humidified 37 oC, 5% CO2 incubator for albumin (200 µg/mL). Blood was collected via jugular venipuncture, permitted to clot at approximately 20°C, and centrifuged, and the serum was aspirated. Serum and saliva samples were stored at –80°C until assayed.

Indirect ELISA was used to determine the feline anti-soy and anti-casein IgG and IgA antibody titers in serum and saliva. Powdered protein isolates were dissolved in 0.2M carbonate buffer (pH, 9.6) to produce a stock solution concentration of 5 mg/mL. Wells were coated with 100 µL of protein solution (5 µg/well) and incubated overnight at 4°C. Following incubation, the contents of the wells were discarded by inversion and the wells were washed with PBSS plus Tween 20 (PBSS + T20; pH, 7.4) for 3 minutes followed by inversion and slapping onto disposable paper towels. Wells were blocked with 150 µL of 1% human serum albumin in PBSS + T20 and incubated for 1 hour at 37°C followed by a washing cycle. Feline serum or saliva (100 µL) optimally diluted in PBSS + T20 was added to the wells and incubated for 2 hours at 37°C followed by a washing cycle. Rabbit anti-cat IgG or anti-IgA horseradish peroxidase conjugate (100 µL), diluted 1:1,000 in PBSS + T20, was added to the wells and incubated for 1 hour at 37°C followed by a washing cycle. Optimal serum, saliva, and reagent antibody dilutions were determined from standard checkerboard dilutions to identify the linear portion of the absorbance-concentration curve. The enzyme substrate was 100 µL of tetramethylbenzidine and hydrogen peroxide, and after 15 minutes, the reaction was stopped with 50 µL of 2M sulfuric acid and optical absorbance read at 450 nm. All ELISA determinations were carried out in triplicate, and data are reported as mean ± SD.

Lymphocyte proliferation assay—Mesenteric lymph node-derived mononuclear cells for proliferation assays were prepared by ficoll-hypaque gradient centrifugation. Lymph node biopsy specimens were broken apart by use of a sterile hypodermic needle, sieved through a cell strainer, suspended in Hank’s buffered saline solution, underlaid with 1077 ficoll-hypaque, and centrifuged for 30 minutes at 900 g at 18° to 20°C with no brake. Harvested mononuclear cells were washed twice in cold Hank’s buffered saline solution, underlaid with 1077 ficoll-hypaque, and centrifuged for 30 minutes at 900 g at 18° to 20°C. The supernatant was then aspirated. Serum and saliva samples were stored at –80°C until assayed.

Results
All cats tolerated the feeding schedule and completed the study as designed. The body weights of all cats remained the same or increased by < 10% during the study. Feces of all cats did not change in appearance during or immediately after the study.

Serum IgG and IgA responses to diet proteins—Serum IgG specific to unprocessed soy or casein was induced by the feeding regime and was detectable from day 7 of feeding and had reached a maximum by day 14 (Figure 1). There was no significant difference in IgG titers against unprocessed casein between the 2 groups. Further dilution of the day 14 and 28 sera to

Figure 1—Mean ± SD serum IgG responses to dietary unprocessed casein (A) or unprocessed soy (B) proteins in cats as detected by indirect ELISA. One group was fed the canned soy diet, followed by an aequous suspension of unprocessed casein protein (open triangles; n = 11); the other group was fed the canned casein diet, followed by an aequous suspension of unprocessed soy protein (closed squares; n = 7). Serum was diluted 1:400, and 5 µg of unprocessed antigen (casein or soy) was bound per well. *Significantly (P < 0.05) different from values for cats fed canned soy diet.

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1:1,600 did not reveal any differences between the groups. There was a difference in IgG production to soy between the 2 groups, with the canned-soy–fed group having a lower response than the unprocessed-soy–fed group on days 14 and 28 ($P = 0.005$ and 0.04, respectively).

Serum IgA responses to unprocessed antigens were also elicited and followed a similar course to IgG (Figure 2). There was no significant difference between the amount of antigen-specific serum IgA from cats fed canned diets and that from cats fed unprocessed proteins.

Comparisons were made between serum IgG and IgA responses on day 28 to unprocessed antigens and canned protein isolates. Cats fed canned proteins did not develop significantly higher antibody responses to canned isolates than cats fed unprocessed proteins for either canned isolate. Cats fed canned soy had a significantly ($P = 0.046$) lower IgG response to the canned isolate than the cats fed raw soy, similar to the responses to the unprocessed soy protein. No significant difference was identified for the serum IgA comparisons.

**Salivary IgA response to diet proteins**—A salivary IgA response to unprocessed proteins was not detected at any time point. However, a significant ($P = 0.006$ and 0.0011 for days 14 and 28, respectively) salivary IgA response to canned casein isolate was detected in those cats fed canned casein but not in cats fed unprocessed casein (Figure 3). An insufficient amount of saliva was available for testing the salivary IgA response to the canned soy isolate.

**Mesenteric lymph node-derived lymphocyte antigen proliferation**—Proliferation of lymphocytes in response to either antigen as detected by incorporation of $^{3}$H-thymidine was slight in both groups, and stimulation indices ranged from 0.9 to 8.2. No significant difference was found between dietary groups at days 7 or 21 to either protein.

**Discussion**

The robust serum IgG and IgA responses to dietary proteins, irrespective of the form the proteins were fed in, were unexpected. This is different from the classical notion of oral tolerance derived from rodent studies and demonstrates the limitations of rodents as models for mucosal immune responses in cats and perhaps other mammals as well, including humans. Results of the study reported here are compatible with results of a study in humans in which B-cell priming occurred following antigen feeding, in contrast with T-cell tolerance. However, even in that study, serum antigen-specific antibodies were not detected until after the subjects were challenged parenterally.

In the study reported here, cats fed the canned soy diet produced less IgG in response to both unprocessed and processed antigens than cats fed unprocessed soy. These results did not support the hypothesis that the cooking process increases the systemic immunogenicity of dietary proteins and are consistent with the concept that the cooking process reduced the number of epitopes. Interestingly, a similar IgG response was not seen in response to canned casein. The difference between canned diets could be due to the increased susceptibility of casein to Maillard compound formation, compared with soy, which may have increased the immunogenicity despite the decreased number of epitopes.

The salivary IgA response to the canned casein isolate provided some evidence to support the hypothesis.
that the canning process increased the immunogenicity of dietary proteins. Although a detectable response was seen in those cats fed the canned casein diet, salivary IgA that reacted with unprocessed casein was not detected. Thus, not only did the canning process create neoantigens from a simple protein mixture, but the immunogenicity of these proteins was increased over their unprocessed native forms. The Maillard reaction that occurs during heating produces insoluble, particulate protein complexes that are less digestible than unprocessed proteins. Suggested mechanisms for increasing the antigenicity in mammals include the melanoidins acting as haptens or increasing the number of undigestible antigens absorbed intact across the intestinal mucosa. Results of a previous study in humans indicate that the Maillard compounds formed from roasting peanuts bind IgE in sera from allergic patients with as much as a 90-fold greater affinity than the unroasted extract. In that study, heat-modified proteins were more resistant to digestion than unheated proteins, supporting the association between protein digestibility and allergenicity. This may be especially pertinent to cats, which have short intestinal tracts suited to highly digestible dietary proteins. Cats are particularly sensitive to the effects of ingested Maillard compounds, which result in small intestinal bacterial overgrowth.

Although identifying the increased ability to stimulate a mucosal IgA response to a dietary protein after canning is a potentially important finding, the clinical relevance of this phenomenon is uncertain. The mucosal IgA response elicited in the cats fed canned casein may be abnormal because no such response to the unprocessed proteins was detected and cats have evolved consuming highly digestible, unprocessed proteins. However, if the response were limited to mucosal IgA production, it would not be predicted to be associated with disease. However, it is conceivable that animals of an atopic genotype may be more likely to produce diet-specific IgE when fed certain poorly digestible, canned diets. In addition, if such poorly digestible diets are consumed during periods of enteritis, this increased immunogenicity may be sufficient to elicit an aberrant clinical response such as food hypersensitivity or diet-responsive inflammatory bowel disease.

Inflammatory bowel disease in cats is clinically confined to the intestinal mucosal immune system. In addition, compartmentalization of lymphocyte trafficking is well recognized in all species that have been studied. Lymphocytes activated within mucosal tissues depart into the systemic circulation, then preferentially reenter mucosal sites via the engagement of mucosal-specific cellular adhesion molecules. Accordingly, it was believed that any abnormally reactive T cells responding to dietary antigens were more likely to be isolated from the mesenteric lymph nodes than peripheral blood. Results of a previous studies indicating antigen-specific proliferation in the mesenteric lymph nodes have generally used T-cell receptor-transgenic mice to ensure a large number of specific clones. The low level of proliferation of lymphocytes in response to raw antigens in these healthy cats is difficult to interpret as evidence of tolerance because it could simply represent the absence of large numbers of specific clones. However, the absence of an IgA response to the unprocessed antigens is consistent with the absence of antigen-specific lymphocyte proliferation. When contrasted with the serum IgG response, the absence of proliferation from mesenteric lymph node-derived lymphocytes provides evidence of compartmentalization of mucosal immune responses in cats, consistent with results in other species. In addition, the systemic humoral responses with absent proliferation from mesenteric lymph node-derived lymphocytes provides an illustration of how food hypersensitivity could cause dermatitis in the absence of clinical gastrointestinal disease, a common manifestation of food hypersensitivity in cats. If the systemic humoral response is skewed toward an IgE isotype, cutaneous mast cell sensitization may develop, whereas the mucosal immune system remains tolerant. However, although it is likely that the serum IgG and IgA are produced in extraintestinal sites, this is not certain.

In the study reported here, the incubation time used in the lymphocyte proliferation assay was determined by results of the first 3 cats that were assayed at 24, 48, 72, 96, and 109 hours, in which 109 hours of incubation time in the presence of antigen gave the highest proliferation, compared with the negative control. Such a prolonged incubation is not without precedent in studies evaluating lymphocyte responses to oral antigens. However, even longer incubation times may have demonstrated notable proliferation.

Due to difficulty producing an intact isolate from the canned diets that was free of endotoxin, we were unable to test for a difference between the canned isolates and the unprocessed antigens in the proliferation assay. In light of the IgA response to the canned casein, the use of the canned antigens in proliferation studies might have provided further support for the hypothesis.

The technique used to isolate proteins from the diets biases the isolate toward unmodified antigens. Large, insoluble particulate antigens and proteins that may be incorporated into lipid emulsions will be largely excluded. Thus, although some difference was observed in the IgA response to the canned isolate, more immunogenic epitopes may have been excluded during the extraction process. This limitation is difficult to overcome given the requirement for aqueous solutions for use in the ELISA protocols or for electrophoresis and western blotting. This technique also prevents evaluation of how different particulate antigens or lipid emulsions may influence in vivo antigen presentation and T-cell stimulation within the intestinal mucosa and associated lymphoid tissues.

Results of the study presented here suggested that robust humoral responses are normal for cats exposed to dietary proteins. However, we did not investigate whether serum immunoglobulin production eventual-
ly decreases with continued presentation in the absence of inflammation—alas, analogous to peripheral self-tolerance. The prevalence of food-specific antibodies in clinically normal individuals of several species suggests that it may not. These responses are similar to those reported in chickens when fed soluble proteins. In chickens, development of oral tolerance is dependent on ingestion of the protein as a powder or particle and is prevented by ingestion of the protein in solution. There may be an important difference between the way proteins in a cat’s natural diet are responded to immunologically and the response to the proteins fed in the study presented here.

If the responses described here are normal, it is unclear how generating an IgG response to dietary proteins could be beneficial. Such antibodies could conceivably be a method for removal of circulating dietary antigens through phagocytosis of opsonized antigens. However, if a sufficient amount of intact dietary protein reaches the systemic circulation, antibody-antigen complexes may develop. The continual antigen exposure that occurs with a consistent diet would then be analogous to the chronic antigenic challenge that can lead to antibody-mediated diseases such as glomerulonephritis, polyarthritis, and thrombocytopenia. The infrequency of these diseases in the general cat population clearly indicates that this does not commonly occur. Low-grade subclinical damage from renal antibody-antigen complex deposition may contribute to the prevalence of chronic interstitial nephritis in cats.

However, consideration should be given to reasons why results of the study reported here may not represent a natural response by cats. Cats have not evolved consuming diets that contain large amounts of a single protein, no matter how digestible. The IgG response may be a reflection of excessive systemic presentation. In contrast, the lesser amounts of single proteins more naturally encountered may not generate such responses. Thus, feeding cats limited antigen diets, rather than complex protein sources, may be detrimental on a population basis. Again, this may be specific to cats or the form of feeding because similar doses of single proteins induce tolerance in dogs and are not associated with serum immunoglobulin responses.

Results of the study presented here indicated that cats develop robust serum IgG and IgA responses to dietary proteins when fed as either aqueous suspensions or as part of canned diets. Additionally, for certain proteins, there may be an increase and a qualitative difference in the immunogenicity of canned diets, compared with the unprocessed proteins. Increased mucosal immunogenicity following the canning process has implications for selection of diets for prevention and management of food hypersensitivity and idiopathic inflammatory bowel disease in cats.

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