

Assessment of Protein Allergenicity on the Basis of Immune Reactivity: Animal Models

Ian Kimber,¹ Rebecca J. Dearman,¹ Andre H. Penninks,² Leon M.J. Knippels,² Robert B. Buchanan,³ Bruce Hammerberg,⁴ Hilary A. Jackson,⁵ and Ricki M. Helm⁶

¹Syngenta Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, United Kingdom; ²Experimental Immunology, Department of Target Organ Toxicology, TNO Nutrition and Food Research, Zeist, The Netherlands; ³Department of Plant and Microbial Biology, University of California Berkeley, Berkeley, California, USA; ⁴Department of Microbiology, Pathology and Parasitology and ⁵Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, USA; ⁶Department of Pediatrics, Arkansas Childrens Hospital Research Institute, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA

Because of the public concern surrounding the issue of the safety of genetically modified organisms, it is critical to have appropriate methodologies to aid investigators in identifying potential hazards associated with consumption of foods produced with these materials. A recent panel of experts convened by the Food and Agriculture Organization and World Health Organization suggested there is scientific evidence that using data from animal studies will contribute important information regarding the allergenicity of foods derived from biotechnology. This view has given further impetus to the development of suitable animal models for allergenicity assessment. This article is a review of what has been achieved and what still has to be accomplished regarding several different animal models. Progress made in the design and evaluation of models in the rat, the mouse, the dog and in swine is reviewed and discussed. *Key words:* allergy, dog, hazard identification, IgE, IgG, immunogenicity, mouse, rat, risk assessment, swine. *Environ Health Perspect* 111:1125–1130 (2003). doi:10.1289/ehp.5813 available via <http://dx.doi.org/> [Online 19 December 2002]

As described elsewhere in this mini-monograph, the first systematic attempt to develop a structured approach to assessment of the allergenic potential of novel food proteins was sponsored jointly by the International Food Biotechnology Council (IFBC) and the International Life Sciences Institute (ILSI) Allergy and Immunology Institute (Metcalfe et al. 1996). The proposed scheme took the form of a decision tree, the starting point for which was determined by the origin of the novel gene and whether or not it derived from a source considered to be allergenic. Among the approaches identified were consideration of the serologic identity of the novel protein with known human allergens; structural similarity to, or amino acid sequence homology with, allergenic proteins; and resistance to proteolytic digestion in a simulated gastric fluid. Although such investigations provide important information and are clearly valuable for the identification of proteins that display serologic or structural homology with known allergens, they do not provide a direct assessment of the inherent sensitizing potential of proteins. For this reason there has been a growing interest in the design, development, and application of appropriate animal models. The consensus at the time of the IFBC/ILSI deliberations was that no appropriate animal models were available. Since then, however, progress has been made, and in 2001 the Food and Agriculture Organization (FAO) and World Health Organization (WHO) convened a special consultation panel to consider food allergenicity

testing and possible revisions to the original IFBC/ILSI decision tree (FAO/WHO 2001). One of the conclusions (conclusion 10) reached by the consultation was that

... animal models have not been evaluated for all food allergens but there is sufficient scientific evidence that using these models will contribute valuable information regarding the allergenicity of foods derived from biotechnology.

This view has given further impetus to the development of suitable animal models, and it is timely now to review what has been achieved and what has still to be accomplished. In this report, progress made in the design and evaluation of models in the rat, the mouse, the dog, and in swine is reviewed and discussed.

Oral and Intraperitoneal Exposure of Brown Norway Rats

Animal models to study the sensitizing potential of new proteins should ideally satisfy several important criteria (Penninks and Knippels 2001; Taylor and Lehrer 1996) that are difficult to attain with a single model. Selection of species and strain, route of exposure for sensitization and challenge as well as the use of adjuvants are important issues to consider. One approach is to use the Brown Norway (BN) strain of rats, as this is a high-immunoglobulin (particularly IgE) responder rat strain. Thus, to a certain degree, this BN rat strain resembles atopic humans in their genetic predisposition to react more readily to

antigens with production of IgE. It has been suggested that for the evaluation of intrinsic allergenic potential of new proteins, oral application is preferred, and that the presence of an adjuvant is to be avoided. BN rats have been used for the study of oral sensitization to food proteins after administration through the diet or by gavage dosing either in the presence (Atkinson et al. 1996) or absence of an adjuvant (Knippels et al. 1998a, 1998b; 1999a, 1999b; 2000).

Studies by Knippels and colleagues used ovalbumin (OVA), a well-defined chicken egg allergen, as a model protein (Knippels et al. 1998b). In these studies not only the dose (0.002–20 mg OVA per dose) but also the mode of application (gavage vs. in the drinking water) and the frequency of application (daily, twice a week, once a week, once every 2 weeks) were investigated (Knippels et al. 1998b). Daily intragastric administration of 1 mg OVA for 42 consecutive days, without the use of adjuvants, resulted in the production of OVA-specific IgG as well as OVA-specific IgE responses in the majority of rats. In general the percentage of IgE responders to OVA exceeded 80%, as measured by both enzyme-linked immunosorbent assay (ELISA) and homologous passive cutaneous anaphylaxis (PCA) assay. Optimal OVA-specific IgE antibody responses were observed around days 28–35. Sometimes, however, no detectable OVA-specific IgE responses were induced upon daily gavage dosing with the same dose (1 mg) of OVA. Generally, using less-frequent administration regimes of 1 mg OVA by gavage did not induce specific IgG or specific IgE antibody responses. Upon exposure in the drinking water, OVA-specific IgG but not OVA-specific IgE antibody was produced (Knippels et al. 1998b).

This article is part of the mini-monograph "Assessment of the Allergenic Potential of Genetically Modified Foods."

Address correspondence to I. Kimber, Syngenta Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire SK10 4TJ, UK. Telephone: 44 1625 515408. Fax: 44 1625 585715. E-mail: ian.kimber@syngenta.com

The authors declare they have no conflict of interest. Received 31 May 2002; accepted 2 October 2002.

Comparative sensitization studies using different strains of rats have been performed to confirm that the BN strain rat is the most suitable choice for these experiments (Knippels et al. 1999b). On the basis of the results of these studies, it was apparent that upon oral exposure of Wistar, PVG, hooded Lister, and BN rats to OVA, only the BN rats developed OVA-specific IgE antibodies, confirming that the BN rat was the most suitable strain for oral sensitization studies.

In subsequent investigations with BN rats, the sensitizing potential of hen egg white (HEW) and cow's milk (CM) proteins was examined. Although antigen-specific IgG responses were found upon daily gavage dosing of the animals with different concentrations of HEW or CM, only a limited number of IgE responders were observed as measured by PCA. However, immunoblotting experiments with these rat sera demonstrated the presence of specific IgE antibodies against both HEW proteins and CM proteins (Knippels et al. 2000). Moreover, both IgG and IgE antibodies present in sera of rats sensitized orally to HEW or CM and in sera of HEW- or CM-allergic patients recognized a comparable profile of allergens in these food products. These results indicate that the specific protein recognition of induced antibodies in the BN rat is comparable with that observed in sera from allergic patients (Knippels et al. 2000).

In addition, BN rats have been sensitized with different doses of either crude raw peanut extract or roasted peanut extract. Dose levels ranged from 0.01 to 10 mg peanut protein extract per day. No clear differences were observed in the sensitizing potency of crude raw peanuts extracts or roasted peanut extracts as measured by Th (T-helper) 2-mediated IgG2a production. Although the number of positive responders increased as the number of daily exposures increased, the magnitude of the IgG2a response was similar in the different dose groups. Only a limited number of animals were IgE-positive as measured by PCA. The IgG2a responses of animals sensitized orally or intraperitoneally with peanut proteins were measured using the three purified major peanut allergens Ara h1, Ara h2 and Ara h3 as substrates. After oral sensitization, IgG2a antibodies were directed against all three major peanut allergens; however, after intraperitoneal sensitization, IgG2a antibodies were directed mainly toward Ara h2.

More recently, the relative allergenicity of selected allergenic and nonallergenic proteins, based on human experience, has been investigated in the BN rat oral exposure model. In these studies Ara h1 purified from peanut, tropomyosin purified from shrimp or beef, and patatin purified from potatoes were evaluated. Preliminary results indicated marked

differences in the two identical sensitization studies performed with these purified proteins in BN rats. Investigators found that the rats in the first study had been unexpectedly pre-exposed in the diet to one allergen used for sensitization and to a cross-reacting allergen, and it is assumed that this affected the results. In the second study the oral sensitizing potential decreased in the following order: Ara h1 > shrimp tropomyosin > patatin, with no sensitization to beef tropomyosin in both studies.

In previous oral sensitization studies with soy proteins, it has been shown that unscheduled dietary preexposure of test animals, or their parental generation, to the antigen under investigation has important influences on the results of oral sensitization studies (Knippels et al. 1998a). Exposure of the parental generation to soy was found to influence the outcome of sensitization studies with the offspring. These studies showed that BN rats bred and raised on a soy protein-containing diet for several generations have soy-specific IgG antibodies. When these rats were fed before breeding with a soy protein-free diet for 6 months, soy protein-specific IgG antibodies were still detectable in the parental animals and also in serum samples collected from the F₁ generation of offspring rats fed on soy protein-free diets for periods up to 6–12 months. In the second, third, and fourth generations of offspring bred on a soy protein-free diet, no soy-specific IgG was detected. Oral sensitization to soy could be achieved in these rats. Therefore, when oral sensitization studies with proteins are performed, at least two generations of animals have to be bred on a diet free of the antigen under investigation to get immunologically naïve and therefore responsive animals.

The BN rat food allergy model was characterized further by Knippels et al. (1999a), who studied immune-mediated effects upon oral challenge of sensitized animals. Local effects induced by oral challenge were studied by measuring gut permeability. Upon an oral challenge with OVA in OVA-sensitized BN rats, gut permeability was increased, as evidenced by an increased uptake of a bystander protein (β -lactoglobulin). In addition to studies on local effects, systemic effects were investigated by monitoring respiratory functions and blood pressure changes (Knippels et al. 1999a). Under the test conditions used, oral challenge with OVA induced only minor effects on the respiratory system or blood pressure in a minority of animals. However, this low incidence is considered to be in accordance with clinical observations in food-allergic patients.

In conclusion the results obtained to date indicate that the BN rat might be a useful animal model in which to study the potential oral allergenicity of novel food proteins

(Knippels and Penninks 2003). However, further testing with either whole food or with additional purified non-, weak-, and strong-allergenic proteins is needed for a more in-depth evaluation of this BN rat model. Moreover, the BN rat model may also be a promising tool to study in more detail various mechanistic aspects of food allergy, which might result in new opportunities with respect to prophylaxis and therapy.

Oral and Systemic Exposure of BALB/c Mice

The Balb/c mouse has also been utilized to evaluate the sensitizing potential of novel proteins. An advantage of this model is that the Balb/c mouse strain is known to favor the development of Th2 type immune responses and the production of IgE antibody.

Although it has been argued that, in the context of assessing the safety of dietary proteins, oral administration is the preferred route of exposure, it has been demonstrated that such a regimen may lack the sensitivity required for effective identification of inherent sensitizing potential. This is probably attributable, at least in part, to the fact that oral exposure may be associated with the development of tolerance (Strobel and Mowat 1998). Consequently, the studies described below focus primarily on systemic (usually intraperitoneal) exposure of mice to the protein of interest.

Using the Balb/c mouse, it is possible to measure the quality and vigor of immune responses after systemic exposure to proteins and to define these proteins as having inherent sensitizing potential if they provoke clear IgE antibody responses. In effect the strategy is to distinguish between immunogenic proteins (that are able to induce specific IgG antibody responses, but not IgE responses) and potentially allergenic proteins (that are able under these conditions to provoke both IgG and IgE antibody production). It has been possible using this approach to demonstrate clear differences between proteins with respect to IgE antibody production. Thus, under conditions of exposure where different proteins elicit largely comparable IgG antibody responses, there can be very substantial variations in their ability to induce specific IgE antibody (Dearman et al. 2000, 2001; Dearman and Kimber 2001; Hilton et al. 1994, 1997). It is assumed that such differences are predicated on the characteristics of the protein that in turn influence the nature of induced immune responses. Among the features that may be important in this respect are the following: size, proteolytic stability, biological function (including enzymatic activity), glycosylation status, overall immunogenicity and the ways in which protein is processed for subsequent presentation to the immune system (Huby et al. 2000; Kimber and Dearman 2001a).

It is important to emphasize that this approach is designed specifically to serve as a tool for hazard identification and characterization (Dearman and Kimber 2001; Kimber et al. 2000; Kimber and Dearman 2001b). It must be acknowledged that proteins defined as having an inherent sensitizing potential as a function of their ability to stimulate IgE responses may not necessarily represent a risk for human health. For instance, a protein with inherent allergenic activity as defined above may fail in humans to cause sensitization when experienced in the diet or if levels of exposure are insufficient to elicit an immune response. Clearly, development of risk assessments for human allergic disease is going to be problematic (not least because of the congenital and acquired factors that influence interindividual differences in susceptibility), but any effective safety assessment process must incorporate a sensitive and selective method for identifying potential hazards. It is our view that this approach employing BALB/c strain mice is currently the best way forward.

The standard protocol used by Kimber and colleagues (Dearman et al. 2001, 2002) can be summarized briefly as follows. Groups of mice (conventionally five per group) receive one of various concentrations of the test protein by intraperitoneal injection. Seven days later this treatment is repeated. At various periods following the initiation of exposure, mice are exsanguinated by cardiac puncture. Individual and pooled serum samples are prepared and stored frozen until analysis. Protein-specific IgG antibody responses are measured using ELISA. For the evaluation of IgE antibody responses, a homologous PCA assay is used. Studies using three proteins of differing sensitizing potential serve to provide an illustrative example of results obtained with this protocol.

Mice were exposed to equal volumes of either 0.2% peanut agglutinin (a minor allergen of the peanut) (Barnett et al. 1983; Burks et al. 1994), 2% ovalbumin (OVA), or a crude potato protein extract (PPE; 10%) containing acid phosphatase activity (and which is assumed to lack significant sensitizing activity). All proteins elicited specific IgG antibody responses that were of maximal vigor 14 days after the initiation of exposure. Under these same conditions of exposure, strong IgE responses were observed 14, 28, and 42 days after exposure of mice to either peanut agglutinin or OVA. In contrast, PPE failed to stimulate high-titer IgE antibody, with only a very weak and transient signal detectable at 28 days.

In separate experiments, responses induced in mice after intraperitoneal administration have been compared with those observed after gavage exposure. In the latter case mice received a daily gavage dose of 1 mg test protein for 28 or 42 days. Results obtained

with OVA are instructive. Although oral dosing resulted in the elicitation of IgG antibody responses in a proportion of exposed mice, only variable and low-grade IgE antibody production was found. This is in contrast to the strong IgE responses observed after systemic exposure of mice to OVA (Dearman et al. 2001). As discussed above, the difficulty in generating robust IgE responses to OVA suggests that for the purposes of hazard identification and assessment of inherent sensitizing activity, systemic exposure may be more appropriate than oral dosing.

Against this background the need now is for a more extensive and more searching evaluation of the accuracy and utility of this approach, and to this end it will be necessary to examine the characteristics of antibody responses induced by a wider range of proteins (including known allergens and proteins known or believed not to cause allergic sensitization). It will be necessary also to determine the reliability and robustness of the method within the context of well-managed interlaboratory collaborative trials. In addition to further examination of this experimental approach for the purposes of hazard identification and characterization, other research objectives are currently being addressed.

The first of these relates to the influence of resistance to proteolytic digestion on sensitizing activity. Previous investigations have indicated that there exists an association between the resistance of proteins to digestion in a simulated gastric fluid and sensitizing activity (Astwood et al. 1996). Although this correlation is not absolute, evaluation of resistance to proteolysis is currently regarded as one component of the safety assessment process (Bannon et al. 2003). It is frequently assumed that such an association reflects the fact that for a protein to induce sensitization after oral ingestion, it will have to display some level of stability in the gastric environment in order to interact effectively with the mucosal immune system. However, it is clear (after gavage exposure at least) that even unstable proteins are able to elicit immune responses in mice when dosed orally (Dearman et al. 2002). On this basis it seems reasonable to suggest that the observed correlation with resistance to proteolysis may not be attributable solely to survivability in the gastrointestinal tract. One hypothesis is that the susceptibility or otherwise to proteolytic digestion may impact on the way in which antigen-presenting cells process proteins for subsequent presentation to the immune system.

A second objective would be to characterize in greater detail the quality of immune responses provoked in mice by protein immunogens and protein allergens. Investigations are focused currently on definition of the cytokine secretion profiles induced

by proteins. Preliminary evidence indicates that, as expected, and consistent with their ability to elicit IgE antibody responses, proteins with known sensitizing potential are associated with a preferential type 2 cytokine expression phenotype. It remains to be seen whether evaluation of cytokine secretion profiles provides an additional or alternative approach to the identification of inherent sensitizing activity.

Assessment of Allergenicity in Dogs I

Thioredoxin reduces disulfide (S–S) groups on proteins to the sulfhydryl state, and researchers have examined whether thioredoxin-induced changes in disulfide proteins affect the recognition of these proteins by IgE antibody. To address this question, studies have been performed using high IgE-producing dogs (Ernel et al. 1997). These animals were descended from a colony of inbred, high IgE-producing spaniel/retriever/basenji dogs maintained at the Animal Resources Service, School of Veterinary Medicine at the University of California, Davis (Davis, California, USA).

On the basis of skin-test challenge of dogs sensitized previously to the native proteins, the disulfide allergens that have been studied show decreased allergenicity upon reduction by thioredoxin, apparently as a result of changes in IgE epitope accessibility. This decrease was demonstrated first with the major allergens of wheat [this decrease was shown with gliadins and glutenins in dogs as well as in humans (Buchanan et al. 1997)] and more recently with the major milk allergen β -lactoglobulin (del Val et al. 1999). Further research with β -lactoglobulin revealed that, in addition to decreasing the skin-test challenge response in animals previously sensitized to the native protein, thioredoxin treatment alleviated the clinical symptoms manifested by the dog (vomiting and diarrhea or constipation). Recently, it has been shown that the thioredoxin-reduced (disarmed) form of β -lactoglobulin can be stabilized by either reoxidation or derivatization with a physiologic disulfide compound (cystamine or oxidized glutathione) (Morigasaki et al. 2001, 2003).

Dogs that were originally sensitized to CM, beef, wheat, and soy proteins were shown to produce specific IgE antibodies and to respond to oral challenges with clinical manifestations similar to those provoked in humans. Additional animals have been sensitized to peanut, walnut, or Brazil nut extracts to determine whether sensitization with these preparations is also accompanied by clinical reactions, and whether cross-reactivity occurs between the different preparations (Teuber et al. 2002). At 6 months of age, these dogs

displayed positive intradermal skin test reactions to these extracts. IgE immunoblotting of sera to peanut, walnut, and Brazil nut showed strong recognition of proteins in the aqueous preparations. At 2 years of age, each of the peanut-sensitized and most of the Brazil nut- and walnut-sensitized dogs reacted on oral challenge with the corresponding primary immunogen. Little or no cross-reactivity occurred among the different preparations measured, either by skin tests or oral food challenges, similar to that in the human clinical picture. Furthermore, on the basis of skin tests and oral challenges, the hierarchy of nut and cereal allergen reactivity was also similar to that seen with humans (peanuts > tree nuts > soy > wheat > barley).

The similarity of the immune response to that observed in humans raises the possibility that the dog could be used to study food allergy and that this model could potentially be used to assess the allergenicity of proteins, including those to which human populations have not been exposed. In addition to being used to assess the allergenicity of the native form of proteins, the dog could in theory predict whether either the introduced protein of interest or an unsuspected host component had become an allergen as a result of the transformation process.

To address this question, pups from three litters were sensitized at birth to a transgenic corn leaf preparation containing a protein of interest that on the basis of digestibility and amino acid sequence analyses was unlikely an allergen. These same pups were sensitized simultaneously to several known allergens (peanut, soy, milk, ragweed extracts). The results obtained over a 2-year period demonstrated that although the known allergens elicited skin-test responses, the protein of interest showed negligible activity. The results support the conclusions that the protein of interest is not an allergen, that the protein had not become an allergen in the transformed leaf, and that unidentified components of the host also had not become allergenic.

Results obtained during the past decade support the use of high IgE-producing dogs as a potential model to assess and predict the importance of proteins as human food allergens. The profile of allergenic foods, response to individual allergens, and clinical reactions of dogs are all similar to those observed in humans. Furthermore, the ranking of the potency of major food allergens is comparable. The dog has additional advantages as an animal model. Its large size permits the performance of gastrointestinal studies such as sampling of mucosa under endoscopy without sacrificing the animal. In addition, as animals can be challenged repeatedly, individuals can be alternately fed comparative diets such as control versus thioredoxin-treated milk preparations.

The dog is, however, not without limitations. Dogs are expensive to maintain, and their immune response (with respect to cytokines and chemokines, for example) is not as well characterized as in models such as the mouse or rat. Furthermore, sensitization is a lengthy process, often requiring up to 18 months of repeated immunization in the presence of adjuvant to achieve a stable response.

In view of its promise, the dog warrants further study as a model to assess and predict potential food allergens. Like other animal models currently being studied (mouse, rat, pig), the dog may offer specific advantages for investigation of this increasingly important problem.

Allergenicity in Dogs II: Spontaneous Food Hypersensitivity/Allergy in a Colony of Dogs

Models used to identify and rank isolated proteins for the potential to induce IgE antibody are highly sensitive for allergenic proteins; however, they may have low specificity for these proteins as dietary components, and these models may not have the power to identify allergens in whole foods. Ideally, to detect foods that are truly allergenic, a model should provide for sensitization by the oral route without adjuvants and demonstrate a clinical response characteristic of atopic disease. Such a model should also manifest IgE responses. It has been suggested that such a model exists in the spontaneously food-allergic dog.

Spontaneous allergic disease in dogs was first reported by Wittich (1941). This report described seasonal rhinitis and conjunctivitis analogous to human hay fever. Later, rhinitis, conjunctivitis, and asthmatic signs were demonstrated after exposing a seasonally spontaneous allergic dog to aerosolized ragweed pollens (Patterson 1960). Although these initial reports in the literature focused on hay fever-like symptoms in dogs, the more common manifestation observed by veterinarians in practice is that of allergic dermatitis, and this was first characterized in detail by Halliwell (1971). From these early observations it was clear that the dog's ability to manifest allergic disease signs similar to humans provided a useful model. Thus, reports have appeared concerning induced allergic disease in dogs (Butler et al. 1983; Gold et al. 1972; Patterson et al. 1974; Peters et al. 1982), and most recently, the induced allergic dog model has been applied to oral sensitization to food proteins (Ermel et al. 1997).

Evidence from more than 30 years of clinical observations shows that spontaneous hypersensitivity to food by dogs is associated with atopic dermatitis (Chamberlain 1978; Criepe 1968; Reedy et al. 1997; White 1986).

Spontaneous food hypersensitivity manifested as dermatitis and enteric signs associated with elevated food allergen-specific IgE has recently been described in the founding stock of a colony of Maltese × beagle dogs. About half of the offspring in this colony manifest clinical signs of atopic disease before 6 months of age when fed standard chow. Additional studies of offspring from this founding stock, reported below, demonstrate the ability of these dogs to be sensitized to allergens present in food, and to respond with clinical signs of atopic disease to food allergens in amounts comparable with those reported to elicit disease in humans. Furthermore, clinical disease is associated with elevated levels of food allergen-specific IgE (Jackson and Hammerberg 2002).

Twelve Maltese × beagle dogs 1–3 years of age, born and reared indoors without exposure to helminth or arthropod parasites, demonstrated clinical signs of nonseasonal atopic dermatitis when fed standard dog chow containing corn, wheat, and soy proteins, among other ingredients (Jackson et al. In press). The dogs were given monthly tablets containing soy and pork for flavoring. The amount of soy and pork protein contained in each tablet provided about 1–2 mg/kg body weight for each dog. Two months before the start of the trial, the diet was changed to a hypoallergenic food consisting of cooked rice and duck, which allowed resolution of clinical signs of atopic dermatitis. On day 0, prior to receiving the flavored tablet, and at regular intervals for 45 days, dogs were bled and scored for pruritus, erythema and excoriation, and evidence of secondary skin/otic infections. These measurements were combined for a total clinical score.

Clinical scores for pruritus showed marked increases at 8–13 days after ingestion of the flavored tablet. Specific IgE antibodies for soy showed statistically significant increases from day 0 levels at day 5 ($p = 0.001$), day 8 ($p = 0.03$), and day 20 ($p = 0.03$). Antibodies for pork tended to be elevated on day 5, but only on day 20 was there a statistically significant increase ($p = 0.03$). Even though corn was not part of the tablet composition, significant increases occurred in anti-corn IgE at the three time points sampled from days 20 to 45. No significant increase was observed in total serum IgE, indicating there was no major polyclonal stimulation of IgE production by the ingestion of this amount of allergen. Thus, the elevated levels of allergen-specific IgE were not due to a nonspecific increase in total serum IgE. Indeed, there was a statistically significant decrease in total serum IgE at day 5.

These results demonstrate that development of clinically significant pruritic skin disease and otitis occurring in Maltese × beagle dogs 2–5 days after being fed small amounts of selected food proteins is associated with

elevated levels of serum IgE specific for these food proteins. A second peak of allergen-specific IgE at about 20 days was also observed. This dynamic response in specific IgE associated with food allergen challenge during maintenance on a hypoallergenic diet can be exploited to identify naturally sensitizing and eliciting allergen epitopes on food allergens.

Peanut Allergy in a Swine Model

Experimentally induced allergic disease in large animals that closely mimics allergic diseases in humans can be a useful model. Investigations in swine and calves demonstrate the induction of hypersensitivity responses that are similar to those of human allergic disease (Barratt et al. 1978). Young piglets have been used as models for sensitization/tolerance to CM and soy proteins, with responses that parallel those seen in young children (Bailey et al. 1993; Hankins et al. 1992; Heppell et al. 1989; Li et al. 1991; Rees et al. 1989; Wilson et al. 1989). It is frequently assumed that similarities exist between swine and humans with respect to skin and gastrointestinal tract anatomy and physiology as well as immunology. The development and characterization of the swine as a model for the allergen-sensitizing capacity of novel proteins will be a valuable tool for investigations into the immunopathologic mechanisms of gastrointestinal allergy.

Although anti-swine IgE is not available commercially, IgE-like responses in swine include *Ascaris suum* airways sensitization and challenge that lead to eosinophil infiltration and a late-phase asthmatic reaction (Alving et al. 1991; Fornhem et al. 1996). Immediate skin-test responses with heat-inactivated serum to antigenic soybean products and *Ascaris suum* extracts have also been described (Barriga and Ingalls 1984; Dreau et al. 1994). Moreover, gastrointestinal-associated adverse immunologic reactions include emesis, diarrhea, bleeding, and weight loss, which are also associated with human gastrointestinal allergy.

Because the newborn piglet mimics many of the features of the human newborn, particularly with respect to digestive and immunologic function, a neonatal swine food allergy model has been developed using peanut as the sensitizing agent. Preliminary experimental designs included intragastric and intraperitoneal sensitizations in the presence or absence of cholera toxin as an adjuvant at different times with oral challenges 2 weeks after the last sensitization. Animals were taken off food and water 18 hr prior to oral challenge. An optimal sensitization regimen consisted of a dose of 500 µg/mL peanut extract administered by intraperitoneal injection in the presence of 100 µg cholera toxin on days 7, 8, and 9, followed

by two additional boosts on days 17 and 25. Using this sensitization regime, greater than 75% of peanut-sensitized piglets responded with clinical symptoms of emesis, extreme lethargy/malaise, tremors, convulsions, and major areas of edematous rashes. The allergic response was determined by repeated intragastric challenge with 10 g peanut meal and skin testing with peanut extracts (whole peanut extract, Ara h1 and 2) performed on alternating weeks beginning at week 5 and ending at week 12.

Physical, histologic, and immunologic assessments were made after oral challenges, skin testing, or tissue analysis. Control animals receiving either phosphate-buffered saline (PBS) or PBS with cholera toxin did not show signs, symptoms, or immunologic or histologic evidence of gastrointestinal allergy. Additionally, peanut-sensitized animals challenged with soybean/peanut-free diet did not respond with allergic symptoms, suggesting a peanut-specific response. The following physical observations were evident in only peanut-sensitized piglets. Whole-body rashes, including maculopapular rashes, lethargy, and difficulty in breathing as evidenced by stridor, were present within 2 hr of oral challenge. Gastrointestinal signs included contractions and emesis within 2 hr of oral challenge and diarrhea within 18 hr. Labored breathing was in evidence and required treatment in two animals.

Immunologic assessment revealed a 2- to 3-fold increase in peripheral blood mononuclear cell (PBMC) proliferation to whole-peanut extract and peanut allergens Ara h1 and 2 compared with PBMC proliferation to medium alone or rice as a control allergen protein. Peanut-specific IgG increased from baseline levels at day 7 (0.1–0.5 µg/mL) to greater than 100 µg/mL by day 40 and persisted to day 90. Immediate skin-test responses as evidenced by wheal and flare reactions suggested an IgE-mediated hypersensitivity response. In PCA experiments, serum from peanut skin-test-positive and oral challenge-positive animals administered intradermally into naïve animals responded with positive skin test to an intravenous challenge of peanut extract 24 hr after intradermal skin sensitization. Heat treatment at 56°C for 1 hr ablated the skin-sensitizing ability, further suggesting an IgE-mediated mechanism.

Animals responding within 2 hr to an oral challenge by emesis were sacrificed for physical and histological assessment of gastrointestinal tract tissues. Dissection of the stomach of these animals revealed minor quantities of peanut meal compared with abundant amounts of peanut meal in the stomachs of nonsensitized or soybean-/peanut-free diet-challenged animals. A summary of histologic data from 6 controls and 17 peanut positive-challenged

animals revealed the following information. The most prominent findings were vascular congestion and hemorrhage that occurred primarily in the proximal intestine. Similar features appeared in other parts of the intestinal tract but did not appear as severe and were probably associated with the early sacrifice of the animals and failure of food to reach the intestinal extremities. Additional markers of a gastrointestinal allergic response included mucus extrusion and submucosal edema. Specifically, the esophagus was routinely normal. The stomach showed normal architecture (10 pigs) focal eosinophilia and submucosal edema (2 pigs), and large amounts of mucus extrusion into the lumen (5 pigs), with vascular congestion and hemorrhage in 2 pigs. In the small intestine the following were observed: focal eosinophilia with large numbers in 2 pigs, submucosal edema (10 pigs), mucus extrusion (2 pigs), epithelial denudation (3 pigs), focal villous shortening (2 pigs), and vascular congestion (14 pigs) with hemorrhage in 3 pigs. The proximal small intestine appeared to be more affected than the distal region. Evidence of food in this region was also limited, suggesting that the early sacrifice may contribute to the limited finding in the distal regions of the small intestine. The colon was normal in 11 pigs, with occasional vascular congestion in 4 pigs; 2 pigs demonstrated crypt abscesses.

In conclusion, the neonatal swine model is an appropriate peanut food-allergy model that can be used to determine the relative physical signs of human allergy including emesis, erythematous rashes, and skin testing. Tissue abnormalities appear to be similar to other endoscopically obtained tissues from allergen-sensitized/challenged animals.

Conclusion

Considerable progress has been made in developing animal models for the assessment of the allergenic potential of novel proteins. However, it is important to acknowledge that none of these has yet been evaluated rigorously or validated formally. Much still has to be achieved, but with a continued investment in relevant research, it should prove possible to contribute to the safety assessment process by providing one or more methods based on responses in animals.

REFERENCES

- Alving K, Matran R, Lundberg JM. 1991. The possible role of prostaglandin D2 in the long-lasting airways vasodilatation induced by allergen in the sensitized pig. *Acta Physiol Scand* 143:93–103.
- Astwood JD, Leach JN, Fuchs RL. 1996. Stability of food allergens to digestion *in vitro*. *Nat Biotechnol* 14:1269–1273.
- Atkinson HA, Johnson IT, Gee JM, Grigoriadou F, Miller K. 1996. Brown Norway rat model of food allergy: effect of plant components on the development of oral sensitization. *Food Chem Toxicol* 34:27–32.
- Bailey M, Miller BG, Teleme E, Stokes CR, Bourne FJ. 1993. Specific immunologic unresponsiveness following active

- primary responses to proteins in the weaning diet of piglets. *Int Arch Allergy Immunol* 101:266–271.
- Bannon G, Tong-Jen Fu, Kimber I, Hinton DM. 2003. Protein digestibility and relevance to allergenicity. *Environ Health Perspect* 111:1122–1124.
- Barnett D, Baldo BA, Howden ME. 1983. Multiplicity of allergens in peanuts. *J Allergy Clin Immunol* 72:61–68.
- Barratt ME, Strachan PJ, Porter P. 1978. Antibody mechanisms implicated in digestive disturbances following ingestion of soya protein in calves and piglets. *Clin Exp Immunol* 31:305–312.
- Barriga OO, Ingalls WL. 1984. Potentiation of an IgE-like response to *Bordetella bronchiseptica* in pigs following *Ascaris suum* infection. *Vet Parasitol* 16:343–345.
- Buchanan BB, Adamidi C, Lozano RM, Yee BC, Momma M, Kobrehel K, et al. 1997. Thioredoxin-linked mitigation of allergic responses to wheat. *Proc Natl Acad Sci USA* 94:5372–5377.
- Burks AW, Cockrell G, Connaughton C, Guin J, Allen W, Helm RM. 1994. Identification of peanut agglutinin and soybean trypsin inhibitor as minor legume allergens. *Int Arch Allergy Immunol* 105:143–149.
- Butler JM, Peters JE, Hirshman CA, White CR Jr, Margolin LB, Hanifin JM. 1983. Pruritic dermatitis in asthmatic basenji-greyhound dogs: a model for human atopic dermatitis. *J Am Acad Dermatol* 8:33–38.
- Chamberlain KW. 1978. Clinical signs and diagnosis of atopic disease in the dog. *J Small Anim Pract* 19:493–505.
- Criep LH. 1968. Allergy in veterinary medicine. *Vet Med Small Anim Clin* 63:855–859.
- Dearman RJ, Caddick H, Basketter DA, Kimber I. 2000. Divergent antibody isotype responses induced in mice by systemic exposure to proteins: a comparison of ovalbumin with bovine serum albumin. *Food Chem Toxicol* 38:351–360.
- Dearman RJ, Caddick H, Stone S, Basketter DA, Kimber I. 2001. Characterization of antibody responses induced in rodents by exposure to food proteins: influence of route of exposure. *Toxicology* 167:217–231.
- Dearman RJ, Caddick H, Stone S, Kenna JG, Basketter DA, Kimber I. 2002. Immunogenic properties of rapidly digested food proteins following gavage exposure of mice: a comparison of ovalbumin with a potato acid phosphatase preparation. *Food Chem Toxicol* 40:625–633.
- Dearman RJ, Kimber I. 2001. Determination of protein allergenicity: studies in mice. *Toxicol Lett* 120:181–186.
- del Val G, Yee BC, Lozano RM, Buchanan BB, Ermel RW, Lee YM, et al. 1999. Thioredoxin treatment increases digestibility and lowers allergenicity of milk. *J Allergy Clin Immunol* 103:690–697.
- Dreau D, Lalles JP, Philouze-Rome V, Touleec R, Salmon H. 1994. Local and systemic immune responses to soybean protein ingestion in early-weaned pigs. *J Anim Sci* 72:2090–2098.
- Ermel RW, Kock M, Griffey SM, Reinhart GA, Frick OL. 1997. The atopic dog: a model for food allergy. *Lab Anim Sci* 47:40–49.
- FAO/WHO. 2001. Evaluation of allergenicity of genetically modified foods. Report of a Joint FAO/WHO Expert Consultation of Allergenicity of Foods Derived from Biotechnology. 22–25 January 2001. Available: <http://www.fao.org/esn/gm/allergygm.pdf> [accessed 11 September 2002].
- Fornhem C, Dahlback M, Kumlin M, Lundberg JM, Alving K. 1996. Effects of local and systemic budesonide on allergen-induced airway reactions in the pig. *Br J Pharmacol* 118:989–997.
- Gold WM, Kessler GF, Yu DY, Frick OL. 1972. Pulmonary physiologic abnormalities in experimental asthma in dogs. *J Appl Physiol* 33:496–501.
- Halliwel RE. 1971. Atopic disease in the dog. *Vet Rec* 89:209–214.
- Hankins CC, Noland PR, Burks AW Jr, Connaughton C, Cockrell G, Metz CL. 1992. Effect of soy protein ingestion on total and specific immunoglobulin G concentrations in neonatal porcine serum measured by enzyme-linked immunosorbent assay. *J Anim Sci* 70:3096–3101.
- Heppell LM, Sissons JW, Banks SM. 1989. Sensitisation of pre-ruminant calves and piglets to antigenic protein in early weaning diets: control of the systemic antibody responses. *Res Vet Sci* 47:257–262.
- Hilton J, Dearman RJ, Basketter DA, Kimber I. 1994. Serological responses induced in mice by immunogenic proteins and by protein respiratory allergens. *Toxicol Lett* 73:43–53.
- Hilton J, Dearman RJ, Sattar N, Basketter DA, Kimber I. 1997. Characteristics of antibody responses induced in mice by protein allergens. *Food Chem Toxicol* 35:1209–1218.
- Huby RD, Dearman RJ, Kimber I. 2000. Why are some proteins allergens? *Toxicol Sci* 55:235–246.
- Jackson HA, Hammerberg B. 2002. Evaluation of a spontaneous canine model of IgE mediated food hypersensitivity: dynamic changes in serum and fecal allergen specific IgE relative to diet change. *Comp Med* 52: 316–321.
- Jackson HA, Jackson MW, Coblentz L, Hammerberg B. In press. Evaluation of the clinical and allergen specific serum IgE response to oral challenge with cornstarch, corn, soy and a soy hydrolysate in dogs with spontaneous food allergy. *Vet Dermatol*.
- Kimber I, Atherton K, Kenna JG, Dearman RJ. 2000. Predictive methods for food allergenicity: perspectives and current status. *Toxicology* 147:147–150.
- Kimber I, Dearman RJ. 2001a. Food allergy: what are the issues? *Toxicol Lett* 120:165–170.
- Kimber I, Dearman RJ. 2001b. Can animal models predict food allergenicity? *Nutr Bull* 26:127–131.
- Knippels LM, Penninks AH. 2003. Assessment of the allergic potential of food protein extracts and proteins on oral application using the brown Norway rat model. *Environ Health Perspect* 111:233–238.
- Knippels LM, Penninks AH, Houben GF. 1998a. Continued expression of anti-soy protein antibodies in rats bred on a soy protein-free diet for one generation: the importance of dietary control in oral sensitization research. *J Allergy Clin Immunol* 101:815–820.
- Knippels LM, Penninks AH, Smit JJ, Houben GF. 1999a. Immune-mediated effects upon oral challenge of ovalbumin-sensitized Brown Norway rats: further characterization of a rat food allergy model. *Toxicol Appl Pharmacol* 156:161–169.
- Knippels LM, Penninks AH, Spanhaak S, Houben GF. 1998b. Oral sensitization to food proteins: a Brown Norway rat model. *Clin Exp Allergy* 28:368–375.
- Knippels LM, Penninks AH, van Meeteren M, Houben GF. 1999b. Humoral and cellular immune responses in different rat strains on oral exposure to ovalbumin. *Food Chem Toxicol* 37:881–888.
- Knippels LM, van der Kleij HP, Koppelman SJ, Houben GF, Penninks AH. 2000. Comparison of antibody responses to hen's egg and cow's milk proteins in orally sensitized rats and food-allergic patients. *Allergy* 55:251–258.
- Li DF, Nelssen JL, Reddy PG, Blecha F, Klemm RD, Giesting DW, et al. 1991. Measuring suitability of soybean products for early-weaned pigs with immunological criteria. *J Anim Sci* 69:3299–3307.
- Metcalfe DD, Astwood JD, Townsend R, Sampson HA, Taylor SL, Fuchs RL. 1996. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Crit Rev Food Sci Nutr* 36:S165–S186.
- Morigasaki S, del Val G, Jung HR, Buchanan BB, Frick OL. 2001. Stabilization of hypoallergenic, hyperdigestible forms of the milk allergen β -lactoglobulin. *J Allerg Clin Immunol* 107:S138.
- Morigasaki S, Jung H, Frick OL, Buchanan BB. 2003. Biochemical basis for hypoallergenic properties of reoxidized β -lactoglobulin. *J Allerg Clin Immunol* 111:S138.
- Patterson R. 1960. Investigations of spontaneous hypersensitivities of the dog. *J Allergy* 31:351–363.
- Patterson R, Mellies CJ, Kelly JF, Harris KE. 1974. Airway responses of dogs with ragweed and ascaris hypersensitivity. *Chest* 65:488–492.
- Penninks AH, Knippels LM. 2001. Determination of protein allergenicity: studies in rats. *Toxicol Lett* 120:171–180.
- Peters JE, Hirshman CA, Malley A. 1982. The Basenji-Greyhound dog model of asthma: leukocyte histamine release, serum IgE, and airway response to inhaled antigen. *J Immunol* 129:1245–1249.
- Reedy LM, Miller WH, Willemese T, eds. 1997. *Allergic Skin Diseases of Dogs and Cats*. Philadelphia:W.B. Saunders.
- Rees AS, Lysons RJ, Stokes CR, Bourne FJ. 1989. The effect of parental immunization on antibody production in the pig colon. *Vet Immunol Immunopathol* 23:171–178.
- Strobel S, Mowat AM. 1998. Immune responses to dietary antigens: oral tolerance. *Immunol Today* 19:173–181.
- Taylor SL, Lehrer SB. 1996. Principles and characteristics of food allergens. *Crit Rev Food Sci Nutr* 36:S91–S118.
- Teuber SS, del Val G, Morigasaki S, Jung HR, Eisele PH, Frick OL et al. 2002. The atopic dog as a model of peanut and tree nut food allergy. *J Allergy Clin Immunol* 110:921–927.
- White SD. 1986. Food hypersensitivity in the dog: 30 cases. *JAMA* 188:695–698.
- Wilson AD, Stokes CR, Bourne FJ. 1989. Effect of age on absorption and immune responses to weaning or introduction of novel dietary antigens in pigs. *Res Vet Sci* 46:180–186.
- Wittich FW. 1941. Spontaneous allergy (atopy) in the lower animal—seasonal hay fever (fall type) in a dog. *J Allergy* 12:247–251.