

Protein Allergenicity: Rat Model

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INTRODUCTION

The possibilities of modern biotechnology have resulted in the introduction of new generations of food and food ingredients and this process is expected to continue further in the near future. In particular, the development and market introduction of genetically engineered food crops has gained much attention in the past few years. For these novel foods, a toxicological evaluation and risk assessment are in most cases major items in their safety evaluation. In food, some proteins (i.e., proteins from peanut, egg-white, tree nuts, fish, etc.) are responsible for the development of food allergy. The potential allergenicity of genetically engineered crops, usually containing novel proteins from nonfood origins, has become an important issue in safety evaluation. There is, however, no universal, reliable, and relevant single test to evaluate the allergenic potency of food products and a case-by-case approach is suggested (1).

The best known allergy approach, in which the source of the gene plays a central role, was developed jointly by the International Biotechnology Council and the International Life Science Institute (ILSI) Allergy and Immunology Institute and published in 1996 (2). This so-called IFBC/ILSI decision tree recommended, based on the data available then, that when transgenic proteins were derived from nonfood sources a careful stepwise process should be followed. Attention should be given to several factors, including amino acid sequence homology and various physicochemical properties such as heat and digestive stability of the protein. For transgenic products derived from known allergenic foods several *in vitro* and *in vivo* test are available, using allergic patients and/or their serum, that will most likely result in a conclusive assessment of its potential allergenicity. Unfortunately,

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from the introduction of this decision tree, it was evident that when the protein of interest does not have a history of dietary exposure or when it has an unknown history of allergenicity, the tests indicated in the decision tree would not be conclusive with respect to the allergenic potential of the protein. Even when negative predictions are observed for all recommended tests, indicating only a low probability that the protein will be a significant food allergen, still no full assurance can be given with respect to the sensitizing potential of the protein studied. Moreover, if a positive prediction is observed for one of the listed parameters, i.e., the digestive stability observed with Cry9C in the Starlink corn, further testing is warranted (3).

Therefore, improvement in the predictive value of the outcome of the decision-tree assessment is needed. This year an FAO/WHO expert consultation proposed a revision to the IFBC/ILSI decision tree strategy and included, among others, additional testing. In addition to the targeted serum testing (screening of serum samples from patients with high IgE levels to the category of organisms to which the source of the protein of interest belongs such as a monocot or dicot plant, a mould, or an (in)vertebrate), animal testing also was proposed (4). However, despite increasing research efforts in recent years, validated and widely accepted animal models are not yet available. Nevertheless, the FAO/WHO expert consultation believes that the animal models in development can provide informative data, although validation will be necessary. An important potential advantage of animal models is the possibility of obtaining information on the sensitizing potency of the new protein(s), relative to known allergens.

In this article, we present a promising model for food allergy research, developed in Brown Norway (BN) rats. The results demonstrate that the BN rats can be sensitized orally to various allergenic food proteins tested, resulting in significant antigen-specific IgE responses, without the use of adjuvants. Upon an oral challenge in previously sensitized animals, local and systemic immune-mediated effects are observed, including decreased breathing frequency and blood pressure, and an increased gastrointestinal permeability.

RAT MODELS

The need for accepted animal models was already clear at the time the ILSI/IFBC decision tree was proposed (2). In the past, several attempts have been made to develop appropriate methods; however, these efforts did not result in the well-validated enteral food allergy models. The unscheduled dietary pre-exposure of the test animals, or their parental generations to the protein under investigation, may have been one of the major factors that negatively affected the results of many oral sensitization studies. Moreover, oral exposure to food allergens may result in induction of immunological tolerance (5, 6) in the three rodent species most frequently used (guinea pigs, mice, and rats).

An animal model should satisfy several important criteria: 1. Sensitization and challenge should preferably be orally, because natural barriers such as the gastrointestinal acid denaturation and enzymatic digestion and mucosal/epithelial layers, which are all known to prevent, reduce, or in any other way influence the allergenicity of proteins should be taken into account (7–10). 2. Preferably, there should be no requirement for adjuvants. Although adjuvant effects may play a role in the sensitization process, it is preferable not to assess the inherent allergenicity of the protein in the presence of adjuvant. 3. The test animal should produce a significant amount of IgE and/or other Th2 specific antibody(sub)classes. 4. The test animal should tolerate most food proteins. 5. Upon a challenge with the allergen, clinical reactions to organ sensitivities should be similar to those seen in humans. 6. Antibody responses should be directed to similar proteins (and allergenic epitopes) in the allergenic food proteins as found in patient sera. 7. The model should be relatively easy to conduct and reproducible in time and in different laboratories.

In rats, the oral sensitization to food proteins has been studied by administration through the diet or by gavage-dosing either in the presence (7, 11) or absence (12–17) of an adjuvant. In this article, the oral sensitization studies performed in BN rats without using an adjuvant are described in more detail (12–16).

FOOD ALLERGY MODEL IN BN RATS

Materials

For sensitization studies, young male Brown Norway (BN) rats were obtained from Charles River (Sulzfeld, Germany) and were 3–5 weeks old at study initiation. During the experiment, and for at least 10 days prior to study initiation, the rats were housed in an animal room maintained at $23 \pm 3^\circ\text{C}$, with a light/dark cycle of 12h, and a relative humidity of 30–70%. The animals were housed in stainless-steel wire cages in groups of 5 or 6 and had free access to food and tap-water. Because hen's egg white (HEW) proteins, as well as purified ovalbumin (OVA) from HEW, cows' milk (CM) proteins, and peanut protein extract (PE) were used for sensitization studies, the rats were bred and raised on a commercially available HEW-, peanut- and CM-protein-free rodent diet (SDS Special Diet Service, LAD1 (E) SQC, Witham, UK).

The total HEW protein extract used in oral sensitization studies was prepared according to the method of Bernhisel-Broadbent et al. (18). In short, a sample of 30g of fresh egg white was added to 300ml of sterile saline and the mixture was rotated overnight at 4°C . The mixture was divided over several tubes and subsequently centrifuged for 10 min at 2500g and 4°C . The tubes were decanted and the supernatant fractions were microcentrifuged for

15 min at 17,000g and 4°C and subsequently filter-sterilized through 0.2 µm filters (Costar Scientific Corporation, Cambridge, USA). Two other antigen sources used were skimmed CM (Albert Heijn, Zaandam, The Netherlands, 4g protein/100ml milk) and OVA (Fluka Chemie, Buchs, Switzerland, purity at 70%). Peanuts from the Runner cultivar (Georgia) were kindly provided by Imko Gelria (Doetinchem, The Netherlands). Peanut extracts were made by mixing 100g ground peanuts with 500ml PBS buffer at room temperature (RT). After 4h stirring at RT, the aqueous fraction was collected by centrifugation (3000g, 30min RT). The aqueous fraction was subsequently centrifuged (8000g, 30min RT) to remove residual traces of fat and insoluble particles. The protein content of the extracts was determined with a BCA protein assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Sensitization Protocol

For oral sensitization animals were exposed by gavage dosing, using a 18-gauge stainless steel animal feeding needle to OVA, HEW-, and CM-proteins ($n = 6$; 0.5, 1, 2.5, 5.0, 10, and 15 mg) or PE-proteins ($n = 10$; 0.01, 0.1, 1, and 10 mg protein/ml tap-water; 1 ml/animal) daily for 6 weeks, without the use of an adjuvant. At weekly intervals blood samples were taken from the orbital plexus under light CO₂ anesthesia or by exsanguination from the abdominal aorta at termination. Sera were prepared and stored at -20°C until analyses for anti-OVA, HEW-protein, CM-protein, PE-protein-specific IgG titers by enzyme linked immunosorbent assay (ELISA) and protein-specific IgE by passive cutaneous anaphylaxis (PCA) test (day 42 sera only).

Measurement of Anti-OVA/HEW/CM/PE Antibodies

ELISA techniques were used to measure IgG class serum antibodies specific for OVA, HEW-, CM-, or PE-proteins. For the detection of antigen-specific IgG, 96-wells microtiter plates (Flat-bottomed, Maxisorp, NUNC, Roskilde, Denmark) were coated overnight at 4°C with 100 µl/well of a 5 µg/ml solution of OVA (Serva, Feinbiochemica, Heidelberg/New York, purity at >98%) or PE-protein solution, or with a 75-µg/ml solution of HEW-proteins or CM-proteins in carbonate buffer, pH 9.6. The plates were washed three times with tap-water containing 0.4% Tween 20 (Merck, Hohenbrunn, Germany). This was followed by the addition of 100 µl/well phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA; Sigma Chemicals Co., St. Louis, MO, USA) and 0.02% Tween 20 (PBS/BSA-Tween 20). After 1h incubation at 37°C, the plates were washed and serial dilutions of rat serum in PBS/BSA-Tween 20 were added to the wells and incubated for 1h at 37°C. After washing, 100 µl/well peroxidase conjugated goat anti-rat IgG (H + L) (Zymed, San Francisco, USA, diluted

1:500) in PBS/BSA-Tween 20 was added. After incubation for 1 h at 37°C, the plates were washed again and an enzyme substrate solution of 3,3',5,5'-tetramethylbenzidine (TMB; Sigma Chemicals Co., 100µl/well; 6mg/ml DMSO) was added. The plates were developed at room temperature for 5 to 15 min. Finally, 100µl/well of 2N H₂SO₄ was added. Optical densities were read spectrophotometrically at 450nm with an ELISA plate reader (Microplate Reader, Bio-rad Laboratories, Richmond, VA, USA). A pool of prebleed sera was used as a negative control. The pooled preserum was measured at a 1:4 dilution. The average extinction in negative control wells, to which three times the standard deviation was added, provided the reference value taken to determine the titer in the test sera. Each test serum was titrated starting at a 1:4 dilution and the reciprocal of the furthest serum dilution giving an extinction higher than the reference value was read as the titer. All analyses were performed in duplicate. Positive and negative control samples were incorporated for each 96-wells plate.

Measurement of Reagenic Antibodies

For PCA-testing we used the method as described by Ovary et al. (19). Naive (untreated) BN rats were shaven on the back and flanks and injected intradermally with 0.1ml of the test sera in serial dilutions, followed 64h later with an intravenous injection of 1ml of a 1:1 mixture of a solution of OVA, HEW-, CM-, or PE-proteins (5mg/ml sterile saline) and a solution of Evans blue dye (2% in sterile saline). After 20–30min the animals were examined for positive responses. The diameter of dye extravasation at the site of the serum injection was measured. The reagenic titer was read as the reciprocal of the furthest dilution giving a colored spot of at least 5mm in diameter. Positive and negative control sera as used in the ELISAs were assayed simultaneously with the test sera on each animal used for the PCA tests.

RESULTS

Antibody Responses to OVA/HEW/CM/PE

Daily intragastric administration of different concentrations of either OVA, HEW, CM, or PE during 42 days, without the use of adjuvants, resulted in antigen-specific IgG, as well as IgE responses. The kinetics of IgG antibody responses induced in BN rats by oral exposure to various proteins in different concentrations were investigated. The optimal dosing concentrations found for the different proteins, based on the height of the induced response and number of responding animals, are presented in Table 1. Oral exposure to 1mg PE by daily gavage dosing during 42 days resulted in

TABLE 1 IgG and IgE Antibody Production Following Oral Exposure of Rats to OVA, HEW, CM, or PE

	² log IgG titer (Day 42)	Number of responders		Induction of response (Day)
		IgG	IgE	
PE (1 mg)	10 ± 1.8	10/10	4/10	7
HEW (5 mg)	7.2 ± 2.5	5/6	1/6	21
CM (10 mg)	2.5 ± 0.7	4/5	1/5	35
OVA (1 mg)	9.5 ± 2.4	6/6	3/6	14

Rats received 1, 5, or 10 mg of OVA, PE, HEW, or CM, respectively, in 1 ml water daily by gavage during 42 days. Blood was sampled weekly and individual sera were tested for specific IgG antibody by ELISA and IgE antibody by PCA. Only day 42 sera were tested for the presence of IgE antibody.

optimal specific IgG and IgE responses as compared with the other dosing groups tested. Administration resulted in very rapid induction of IgG antibody (day 7; ²log titer 5.7 ± 0.6 with 3 of 10 animals responding) with maximal responses provoked within 21–28 days. In 40% of these animals, specific IgE responses could be detected at day 42. Administration of doses as low as 0.01 mg PE by daily gavage during 42 days resulted in the induction of IgG antibody at day 7 (²log titer 4 ± 0 with 2 of 10 animals responding). Exposure to 1 mg OVA by daily gavage dosing resulted in optimal responses compared with the other dosing groups tested. At day 42, specific IgE could be detected in 50% of the animals, which was low compared with the number of responding animals observed in other studies conducted with OVA (>65%).

The kinetics of the IgG response were somewhat delayed compared with the responses induced by PE. The onset of IgG antibody production was observed at day 14 (²log titer 6.8 ± 3.1 with 4 of 6 animals responding) with optimal responses within 28–35 days. For the induction of maximal responses to both HEW and CM higher doses of protein were needed, 5 mg and 10 mg protein by daily gavage dosing during 42 days, respectively. In both cases not all animals developed a specific IgG response, whereas 1 animal in each group showed a positive PCA reaction. Although in both groups only 1 animal developed specific IgE as measured by PCA testing, immunoblotting experiments using the same sera indicated a much higher number of IgE-positive animals (16). Maximal responses against HEW were provoked within 42 days of initiation of exposure and detectable IgG antibody observed within 21 days of treatment (²log titer 4 ± 0 with 2 of 6 animals responding). The vigor of the induced IgG antibody response was lower as compared with animals exposed to PE and OVA, but higher compared with animals exposed to CM. For CM, maximal responses were

observed at day 42 of treatment and detectable IgG antibody observed within 35 days of exposure ($^2\log$ titer 2.5 ± 0.7 with 2 of 5 animals responding). The induced IgG antibody response was much lower compared with the other proteins tested.

CONCLUSIONS

It is evident that new approaches are needed to appropriately assess the potential oral allergenicity of "novel proteins." The development of a predictive animal model is of major importance and has been proposed in the new FAO/WHO 2001 decision tree (4). In particular, if transferred genes, coding for new proteins, are derived from products with an unknown history of allergenicity or if proteins show one or more physico/chemical characteristics of known allergens, the ultimate proof for presence/absence of sensitizing activity can be addressed in an appropriate animal model. In this article, we have summarized the development of an oral sensitization protocol in which without an adjuvant BN rats are sensitized to various allergenic food proteins (12–16). Recently, these results have been confirmed for OVA by Akiyama et al. (17), although other researchers could not induce specific IgE after gavage dosing BN rats (20). The possible occurrence of systemic effects upon an oral challenge were investigated by monitoring respiratory functions and blood pressure. An oral challenge with OVA did not induce a clear effect on the respiratory system or blood pressure in the majority of the animals. However, some animals demonstrated temporary decrease in breathing frequency or systolic blood pressure (15), which is in accordance with the human clinical practice. Following the studies on systemic effects, the occurrence of local effects after an oral challenge was demonstrated by an increased gut permeability measured by an increased uptake of the bystander protein β -lactoglobulin 1.5 hours after an oral challenge (15).

The results presented here indicate clearly that protein extracts of allergenic foods, or a purified allergenic protein, when administered by gavage were immunogenic in BN rats inducing IgG antibody responses. Although not as pronounced as the induced IgG responses, oral exposure also induced specific IgE to the food proteins tested in a proportion of the exposed animals. The data reveal that significant differences appear to exist between the proteins examined with respect to IgE and IgG antibody production. Based on the number of IgE responding animals, the magnitude and kinetics of the IgG antibody response, using the optimal dosing concentration of the protein extracts of allergenic foods, the best response was observed in animals treated with PE followed by HEW and CM. Because OVA, in contrast to the other allergenic food extracts tested, is a purified protein, it is difficult to compare directly OVA results with the other test groups with respect to ranking.

The observed pattern of response using allergenic foods appears to reflect what is known of the allergenic properties of these proteins to cause sensitization in humans. Although it is difficult to determine a rank order, the obtained results indicate that PE is more immunogenic/allergenic compared with HEW followed by CM. Animals exposed to 1 mg PE developed the highest IgG antibody response with rapid kinetics and 40% of the animals developed a specific IgE response. The amount of proteins administered to the animals treated with HEW and CM must be higher to obtain maximum responses, 5mg and 10mg, respectively. In addition, not all animals developed an IgG antibody response with delayed kinetics and only 1 animal developed an IgE response.

Although additional studies with more proteins, either as whole foods or as purified strong-, weak-, and nonallergenic purified protein, are needed to validate further this BN rat model, the results to date indicate that the BN rat might be a useful and predictive model to study the potential oral allergenicity of "novel" food proteins. In addition, when using an animal model to screen for the potential allergenicity of food proteins, the data summarized above indicate the need to test different dose levels of the food protein to obtain an appropriate rank order activity.

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