

Determination of protein allergenicity: studies in rats

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Abstract

For the safety evaluation of genetically engineered crops the potential allergenicity of the newly introduced protein(s) has become an important issue. There is, however, no universal and reliable test system for the evaluation of the allergenic potency of food products. The best known allergy assessment proposal is the careful stepwise process using the IFBC/ILSI decision tree. Unfortunately, the described tests are not always conclusive, especially if the gene source coding for the protein has no history of dietary use and/or an unknown history in terms of allergenicity. The further testing warranted should in particular be focused on the prediction of the sensitizing potential of the novel protein, for which animal models are considered to be needed. In this paper the results are summarized of a promising food allergy model developed in Brown Norway (BN) rats. The results demonstrate that BN rats can be sensitized orally to the various allergenic food proteins tested, resulting in significant antigen-specific IgE responses, without the use of adjuvants. Upon oral challenge of previously sensitized animals, local and systemic immune-mediated effects, such as increased gastrointestinal permeability and decreased breathing frequency and blood pressure, could also be observed. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Advances in modern biotechnology techniques will result in the introduction of an increasing number of new generations of food and food ingredients. In particular, the development and market introduction of genetically engineered food crops has gained considerable attention in the past few years. A large number of different plant species, including the most economically

important crops like soy, corn, canola, cotton etc., have been successfully genetically engineered in the past few years, of which only a few have reached the marketplace. A toxicological evaluation and risk assessment are, in most cases, major items in the safety evaluation of novel foods and novel food ingredients.

As some proteins in our food are responsible for the development of food allergy and most of the genetically modified foods developed up to now contain new proteins, their safety evaluation should include an assessment of the allergenicity of such a new protein. Although, however, the potential allergenicity of foods

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derived from genetically engineered crops has become an important issue in their safety evaluation, there is no universal, reliable and relevant test for evaluation of the allergenic potency of food products and a case-by-case approach is required (Wal, 1999). The best known allergy assessment proposal is the careful step-wise process using a decision tree strategy as suggested by the IFBC/ILSI (Metcalf et al., 1996).

The origin of the gene is of great importance with respect to the routing within this decision tree. Particularly in cases where the source of the protein of interest does not have a history of dietary exposure or has an unknown history in terms of allergenicity, the tests indicated in the decision tree are not conclusive. The evaluation of any allergenicity of the molecule introduced or expressed in these foods is a complex but vital question. As historical, clinical and epidemiological data are generally scarce it is therefore necessary to turn to indirect methods to evaluate the possible allergenicity, as listed in the ILSI/IFBC decision tree.

These include structure analysis and amino acid sequence comparison, study of physical and chemical properties like digestive and process (heat) stability, and consideration of the amount of the protein in the foods and consumption patterns. Unfortunately, these tests are not conclusive with respect to the potential allergenicity of the protein involved. When negative predictions are obtained in all of the recommended tests, there is a low probability that the protein is a significant food allergen, but still no full assurance can be given with respect to the sensitizing potential of the protein studied. If a positive prediction is obtained for one of the listed parameters, further testing is warranted and this should be focused, in particular, on the prediction of the sensitizing potential of the novel protein. However, a universal, reliable and relevant *in vitro* or *in vivo* test to assess the sensitizing potential of a new protein is not available. Because of the complexity of the immune system, and because different factors are known to influence sensitization in susceptible individuals, it is not expected that this process can be studied *in vitro*. Therefore, to determine the sensitizing potential

of proteins, the most direct approach will be the development and validation of a widely accepted animal model. An important advantage of animal models may turn out to be the possibility of obtaining information on the potential allergenicity of proteins, in addition to information on their potency relative to well-known allergens.

1.1. Animal models in research on allergenicity of food proteins

Because of the limitations of the approaches described above and the restricted possibilities for research involving human subjects, animal models suitable for research on the allergenicity of food proteins are considered to be of real value. Although much research is being devoted to developing suitable animal models that mimic the antibody response, or even the symptoms upon a challenge reaction like that observed in human allergic patients, no validated animal model exists (Taylor and Leher, 1996).

In the past, several attempts have been conducted to develop animal models for food allergy research. These efforts, however, hardly resulted in structured approaches aimed at the development of well-validated enteral allergenicity models. For food allergy research, three rodent species have frequently been used, viz. guinea pigs, mice and rats. Occasionally also other species like the dog (Ermel et al., 1997) are used. Guinea pigs have frequently been used for oral sensitization studies to food proteins (Devey et al., 1976; Piacentini et al., 1994) and found to be very sensitive. Currently, they are still used for testing of, amongst others hypoallergenic infant formulas or other formulas based on modified (hypoallergenic) proteins. Drawbacks for the further use of the guinea pig in food allergy research are the significant differences in immunophysiology when compared with other species, the limited knowledge of its immune system, the lack of tools to study its immune system, and its questionable specificity in allergic sensitization.

In the past, studies in mice on oral protein administration (diet/gavage/drinking water) without adjuvants readily resulted in tolerance induction (Strobel and Ferguson, 1984; Mowat, 1987).

More recent studies in mice, using repeated enteral protein administration in combination with adjuvants, showed that immune priming or sensitization can be achieved (Challacombe and Tomasi, 1980; Strobel and Ferguson, 1986; Ito et al., 1997; Li et al., 1999, 2000). For assessment of potential allergenicity, the intraperitoneal route of exposure is also studied in mice (Hilton et al., 1994, 1997; Dearman et al., 2000), focusing on both antigenicity and allergenicity of new proteins. For the rat, oral sensitization to food proteins administered through the diet or by gavage-dosing in the presence (Bazin and Platteau, 1976; Atkinson et al., 1996) or absence (Knippels et al., 1998a,b, 1999a,b, 2000) of an adjuvant has also been reported. Some advantages of the rat as a species for oral allergy research are: the rat is the most commonly used species in toxicity testing so the oral sensitizing properties of a protein can be evaluated in perspective with the other information obtained on the health effects, there is a reasonable amount of knowledge on the rat immune system, and many tools are available for immune related studies. As considerable variations exist between the different approaches followed in the different species there is an urgent need for the development of (a) predictive, validated and accepted animal model(s) to identify the potential allergenicity of novel proteins.

1.2. Criteria for an ideal animal model

Although animal models are considered to provide important information on fundamental questions concerning sensitization and mechanistic research on allergy, no well-validated models exist for assessing the allergenic potential of specific proteins in humans. As also indicated by Taylor and Leher (1996), such an animal model should satisfy several important criteria, including: (1) sensitization and challenge should preferably be orally — because natural barriers such as gastrointestinal acid denaturation and enzymatic digestion and the 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 (Pauwels et al., 1979; Strobel and Ferguson, 1984; Turner et al., 1990;

Atkinson et al., 1996); (2) preferably there should be no use of adjuvants — although adjuvants in real life situations may play a role in the sensitization process, it is preferred to assess the inherent allergenicity of the protein; (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 an allergen, clinical reactions with respect to organ sensitivities should be similar to those seen in humans; (6) antibody responses should be directed to similar proteins in the allergenic food as found in patient sera; and finally, (7) the model should be relatively easy to conduct and reproducible over time and in different laboratories.

1.3. Food allergy model in Brown Norway rats

Although immunological tolerance induction may occur in rats upon oral antigen feeding (Steinmann and Wottge, 1990), it has not been observed to be the general response. However, considering also the limitations of guinea pigs and mice, the rat was considered to be (the most) promising species for the development of an oral feeding protocol. To sensitize rats orally to food proteins we started our studies with Brown Norway (BN) rats. This is a high-immunoglobulin (particularly IgE) responder rat strain and thus, to a certain degree, resembles atopic humans in their genetic predisposition to react with an overproduction of IgE to antigens. In our first studies, ovalbumin (OVA), a well defined chicken allergen, was used as a model allergen. Apart from using different sensitization protocols in these first studies, OVA was also administered either via the drinking water or by gavage. Only in the case of daily oral gavage dosing were the animals successfully sensitized (Knippels et al., 1998a).

To study if, indeed, the BN rat is the preferable rat strain, comparative sensitization studies were performed using different strains of rats. Based on the results of these studies, it was evident that upon oral exposure of Wistar, PVG, Hooded Lister and BN rats to OVA, only the BN rats developed OVA-specific IgE antibodies, confirming the choice of the BN rat for oral sensitization

studies (Knippels et al., 1999a). The selectivity of the antibody responses in the BN rat (IgE) to hen's egg and milk proteins was studied with serum of sensitized BN rats and that of patients allergic to these allergens. The antibodies in the two species were found to be directed to the same proteins in these allergenic foods (Knippels et al., 2000).

One important factor that may have negatively affected the results of many oral sensitization studies could have been the unscheduled dietary pre-exposure of test animals or their parenteral generation to the antigen under investigation. As shown in the sensitization studies with soy, exposure of the parental generation to the antigen under investigation will indeed influence the outcome of sensitization studies with the offspring (Knippels et al., 1998b). Therefore, when oral sensitization studies with proteins are performed, one has to ensure that at least two generations of animals have been bred on a diet free of the antigen under investigation to obtain immunologically naïve animals.

The BN rat food allergy model was further characterized by studying immune-mediated effects upon an oral challenge, showing effects on breathing frequency, gastrointestinal permeability and blood pressure (Knippels et al., 1999b). Taken together, these observations suggest that this BN rat model might provide a suitable animal model to study the allergenicity of food proteins, although further validation of the model is needed.

2. Methods

2.1. Rats and materials

For the sensitization studies, young male BN rats were obtained from Charles River (Sulzfeld, Germany) and were 4–6 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 12 h, and a relative humidity of 30–70%. The animals were housed in stainless-steel wire cages in groups of four or five and had

free access to food and tap-water. As hen's egg white (HEW) proteins, as well as purified OVA from HEW and cow's milk proteins (CM) were used for sensitization studies, the rats were bred and raised on a commercially available HEW-protein and CM-protein free rodent diet (SDS Special Diet Service, LAD1 (E) SQC, Witham, UK). To ensure the animals used were immunologically naïve with respect to the antigens under investigation, pre-study blood samples were always tested for HEW-protein and CM-protein specific antibodies.

The total hen's egg white (HEW) protein extract (total protein content 24 mg/ml) used in oral sensitization studies was prepared essentially according to the method of Bernhisel-Broadbent et al. (1994). In short, a sample of 30 g of fresh egg white was added to 300 ml 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 $2500 \times g$ and 4°C . The tubes were decanted and the supernatants were micro-centrifuged for 15 min at $17\,000 \times g$ and 4°C and subsequently filter-sterilized through $0.2\ \mu\text{m}$ filters (Costar Scientific Corporation, Cambridge, USA). The protein content of the extract was determined by BCA protein assay (Pierce, Rockford, USA) according to the manufacturer's instructions. The two other antigen sources used were skimmed CM (Albert Heijn, Zaandam, The Netherlands; 4 g protein/100 ml milk) and OVA (Fluka Chemie, Buchs, Switzerland; purity: 70%) and skimmed cow's milk (CM; Albert Heijn, Zaandam, The Netherlands; 4 g protein/100 ml milk).

2.2. Sensitization protocol

For oral sensitization, the animals were exposed by gavage dosing, using a 18-gauge stainless steel animal feeding needle, to OVA, HEW-proteins, or CM-proteins (1 or 10 mg protein/ml tap-water; 1 ml/animal) for 6 weeks, without the use of an adjuvant. At weekly intervals, blood samples were obtained from the orbital plexus under light CO_2 anaesthesia or by exsanguination from the abdominal aorta after death. After coagulation for 1 h at room temperature, the blood samples were

centrifuged (Heraeus Minifuge T, Osterode, Germany) for 20 min at $2000 \times g$ and 4°C to obtain sera. The sera were stored at -20°C until analyses for anti-OVA, HEW-protein and CM-protein specific IgG titers by Enzyme Linked Immunosorbent Assay (ELISA) and anti-OVA, HEW-protein and CM-protein specific IgE by ELISA and passive cutaneous anaphylaxis (PCA)-test.

2.3. Measurement of anti-OVA/HEW/CM antibodies

ELISA-techniques were used to measure serum antibodies specific for OVA, HEW-proteins or CM-proteins. For the detection of OVA-specific IgG, 96-wells microtiter plates (Flat-bottomed, Maxisorp, NUNC, Roskilde, Denmark) were coated overnight at 4°C with $100\ \mu\text{l/well}$ of a $5\ \mu\text{g/ml}$ solution of OVA (Serva, Feinbiochemica, Heidelberg/New York, purity: $>98\%$) or with a $75\ \mu\text{g/ml}$ solution of HEW-proteins in carbonate buffer, pH 9.6 or with CM-proteins. The plates were washed three times with tap-water containing 0.4% Tween 20 (Merck, Hohenbrunn, Germany). This was followed by the addition of $100\ \mu\text{l/well}$ phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA; Sigma, St. Louis, MO) and 0.02% Tween 20 (PBS/BSA-Tween 20). After 1 h 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 1 h at 37°C . After washing, $100\ \mu\text{l/well}$ 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, $100\ \mu\text{l/well}$; $6\ \text{mg/ml}$ DMSO) was added. The plates were developed at room temperature for 5–15 min. Finally, $100\ \mu\text{l/well}$ of $2\ \text{N}\ \text{H}_2\text{SO}_4$ was added. Optical densities were read spectrophotometrically at $450\ \text{nm}$ with an ELISA plate reader (Microplate Reader, Bio-Rad Laboratories, Richmond, USA). A pre-sera pool was used as negative control. The pooled pre-serum was measured at a 1:4 dilution. The average extinction in negative control wells, to which three times the stan-

dard 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.

For the detection of OVA-specific IgE, 96-well microtiter plates were coated overnight at 4°C with $100\ \mu\text{l/well}$ mouse anti-rat IgE (MARE-1, Zymed) at a concentration of $1.5\ \mu\text{g/ml}$ in carbonate buffer, pH 9.6. The plates were washed and $100\ \mu\text{l/well}$ of PBS/BSA-Tween 20 was added. After incubation for 1 h at 37°C , the plates were washed, and diluted rat serum samples were added and incubated for 2 h at 37°C . The plates were washed and subsequently $100\ \mu\text{l/well}$ of a $1\ \mu\text{g/ml}$ solution of an OVA-digoxigenin (DIG) conjugate was added. The DIG was obtained from Boehringer (Mannheim, Germany) and coupling to OVA was performed according to the manufacturer's instructions. The labelled OVA was separated on a sephadex G-25 column (Boehringer) and labelling efficiency was determined spectrophotometrically at $280\ \text{nm}$. Incubation with OVA-DIG was performed for 1 h at 37°C . After washing, $100\ \mu\text{l/well}$ peroxidase conjugated sheep anti-DIG Fab fragments (Boehringer, Mannheim, Germany) diluted 1:3000 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 TMB was added. Plate development, measurement and titer elaboration were as described for the OVA-specific IgG ELISA.

2.4. Measurement of reaginic antibodies by passive cutaneous anaphylaxis (PCA)

For PCA-testing the method as described previously by Ovary (1964) was used. Naïve (untreated) BN rats were shaven on the back and flanks and injected intradermally with $0.1\ \text{ml}$ of the test sera in serial dilutions, followed 64 h later with an intravenous injection of $1\ \text{ml}$ of a 1:1 mixture of a solution of OVA, HEW-proteins or

CM-proteins (5 mg/ml sterile saline) and a solution of Evans blue (2% in sterile saline). After 20–30 min, 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 coloured spot of at least 5 mm in diameter. Positive and negative control sera as used in the ELISAs were assayed simultaneously with the test serum on each animal used for the PCA tests.

2.5. Measurement of gut permeability

In order to study possible changes in gut permeability in sensitized rats, the uptake of a bystander protein, viz. β -lactoglobulin, was followed with time after an oral challenge. Therefore, OVA-sensitized and control animals were challenged orally with 2 ml of a 50 mg/ml OVA-solution in tap-water or 2 ml of tap-water, respectively. Thirty minutes after the oral challenge with OVA, the animals received an additional intra-gastric dose of β -lactoglobulin (β -LG, 1 ml of a 100 mg/ml solution in tap-water; obtained from Sigma; purity: 90%). At 0.5, 1, 2, 3 and 5 h after the β -LG administration, blood samples were collected from the orbital plexus under light CO₂ anaesthesia or after death 8 h after the β -LG administration by exsanguination from the abdominal aorta. The collected sera were prepared, stored and used for the quantification of β -LG by ELISA as previously described (Knippels et al., 1999b).

2.6. Measurement of blood pressure

In order to determine possible changes in blood pressure, rats were trained, during the sensitization period, to get used to the measurement equipment in order to avoid stress during the experiments. Before challenge, individual baseline blood pressures of the animals were determined on two separate days. Prior to blood pressure measurements, the animals were placed under a heating light (30–32°C) for 20 min. Thereafter, they were restrained in a small tube that was maintained at 20°C. An inflatable pressure cuff

was put around the tail and a distal taped sensor was used to record the systolic blood pressure. Sensor and cuff were controlled by a sphygmomanometer (Nacro Biosystems, type PE-300) which was connected to a reader (Nacro Biosystems, Type MK-II). The OVA sensitized and control animals were challenged orally with 2 ml of a 5 mg/ml OVA-solution in tap-water or 2 ml of tap-water. Blood pressures were recorded at intervals over a period of 7 h.

2.7. Measurement of respiratory function

To measure respiratory frequency, assessed in a plethysmograph with a separate head and body chamber and matched pressure transducers, rats were restrained in modified Batelle tubes (Batelle, Geneve, Switzerland). To give an airtight seal between head and neck at the one side and thorax and abdomen at the other, the tubes had a water-wetted silicone diaphragm. The tube was placed in the body chamber with the open end of the tube fitting into the front chamber. Each plethysmograph was provided with a pressure transducer which sensed changes created by inspiration and expiration and transmitted amplified signals to a polygraph recorder, so allowing determination of respiratory frequency and pattern. Breathing frequencies were determined by means of recording the pressure signal in the volume-calibrated body chamber. Animals sensitized with OVA were challenged orally with 2 ml of a 10, 25, 40 or 50 mg/ml OVA-solution in tap-water. Control animals received an oral challenge with 2 ml of a 10, 40 or 50 mg/ml OVA-solution in tap-water. Prior to challenge, the respiratory function was measured constantly for 15 min and immediately upon an oral challenge with OVA constantly over the first 10 min and thereafter for periods of 30 s, once every 5 min for a total period of 6 h.

3. Results

3.1. Antibody responses to OVA/HEW/CM

Daily intra-gastric administration of 1 mg OVA for 42 days, without the use of adjuvants, resulted

in antigen-specific IgG as well as IgE responses in almost all rats. In general, the percentage of IgE responders in the studies exceeded 80%, as measured by ELISA and PCA. Optimal specific IgE antibody responses were observed around day 28–35. Occasionally, however, no OVA-specific IgE responses were induced upon daily gavage dosing with OVA in the studies. Using less frequent administration regimens of 1 mg OVA by gavage in general did not induce specific IgG or specific IgE antibody responses. Upon ad libitum exposure, only OVA-specific IgG but no OVA-specific IgE was produced (Knippels et al., 1998a).

Although antigen-specific IgG responses were found upon daily gavage dosing of the animals to 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 specific-IgE antibodies against both HEW-proteins and CM-proteins (Knippels et al., 2000). Moreover, antibodies present in sera of orally sensitized rats to HEW or CM and in sera of allergic patients to HEW or CM recognized a comparable profile of allergens in these allergic food products. Specific IgG antibodies in sera from both hen's egg-allergic patients and rats exposed orally to HEW-proteins recognized a whole series of proteins, although the profile appears to be the same. Despite the fact that egg white is a complex mixture of more than 20 proteins, the specific IgE antibodies in sera from both hen's egg-allergic patients and rats exposed orally to HEW-proteins recognized the same proteins, viz. ovotransferrin, ovalbumin, ovomucoid and, to a lesser extent, lysozyme, which are claimed to be the major allergens for hen's egg allergic humans (Ebbehøj et al., 1995). No reaction was observed against any other protein present in the HEW-protein extract.

The same phenomenon was observed when the pattern of protein recognition by antibodies in sera from rats sensitized orally to CM-proteins and antibodies present in sera from milk allergic patients were compared. Although cow's milk contains more than 30 proteins, the induced antibodies were directed mainly against β -LG and, to a lesser extent, against the caseins, whereas no

reaction was observed against any other protein present in CM.

These studies indicate that upon daily intra-gastric dosing with HEW-proteins or CM-proteins, the specific protein recognition of induced antibodies in the BN rat is comparable to that observed in sera from allergic patients. The same phenomenon was described in BN rats sensitized intraperitoneally with CM which produced a profile of IgE antibodies to milk proteins similar to that observed in humans (Atkinson et al., 1996). Although, the induced antibodies in the BN rat apparently react to relevant proteins compared to the human situation, it remains to be elucidated whether the induced specific antibodies in the rat react to the same epitopes as the antibodies in the sera from patients. Furthermore, it should be recognized that the differences in responses to the different food proteins observed in experimental animals as well as in patients will probably be due to a combination of factors such as the dose of allergen, known to influence the outcome of oral sensitization, and the allergenicity of the proteins used (Pauwels et al., 1979; Strobel and Ferguson, 1984; Turner et al., 1990; Atkinson et al., 1996). However, the same allergens were recognized when compared to food allergic patients and no antibodies were produced to the majority of other proteins present in CM and HEW-protein extract.

These results indicate that enterally exposed BN rats and young patients demonstrate IgE antibody responses to a comparable selection of proteins upon exposure to different protein mixtures and further support that the BN rat may provide a suitable animal model for assessing the allergenicity of (novel) food proteins and for research on mechanisms of food allergy. For a more detailed characterization of the rat model developed, additional studies were performed in OVA sensitized rats to study local and systemic immune-mediated effects upon an oral challenge with OVA (Knippels et al., 2000).

3.2. Local and systemic challenge effects

Upon renewed oral antigen exposure of food allergic patients many different clinical signs or

physiological reactions may occur, ranging from oral allergy syndrome, effects on gastrointestinal tract physiology, the respiratory and/or cardiovascular system to effects on the skin (Bruijnzeel-Koomen et al., 1995). In the BN rat model, oral challenge reactions should also be studied to show if, as in allergic patients, clinical reactions can be induced and, as in patients, involve various organs. Therefore, the animals previously sensitized with OVA were challenged orally 10 days after the oral sensitization period by intra-gastric intubation with doses of OVA varying between 10 and 100 mg. Subsequently effects on gastrointestinal permeability, blood pressure and breathing frequency were investigated (Knippels et al., 1999b).

The possible occurrence of local effects upon an oral challenge were studied by measuring gut permeability. Upon an oral challenge with OVA, gut permeability was increased as evidenced by an increased uptake of a bystander protein (β -LG). One hour after an OVA challenge followed by a dose of β -LG 30 minutes later, the amount of β -LG in the sera of previously sensitized rats was significantly higher compared to non-sensitized animals. Several models of intestinal hypersensitivity to food proteins have shown that antigen challenge of the sensitized intestine causes alterations in ion transport, permeability and motility (Crowe and Perdue, 1992; Berin et al., 1997). Also, mediators such as histamine, platelet-activating factor, prostaglandins, leukotrienes and some newly formed cytokines release in anaphylactic reactions have been shown to alter mucosal function in experimental models (Heyman et al., 1994; Kanwar et al., 1994). Whether the increased macromolecular passage is due mainly to transcellular or paracellular transport is still not clear. However, Scudamore et al. (1995) showed that the release of rat mast cell protease-II, a known rat mucosal mast cell mediator, increases epithelial permeability via a paracellular route. Together with the finding that a significant amount of intact β -LG is present in sera of sensitized animals, this may suggest that the epithelial permeability is increased in our animals via the paracellular route, although an increased permeability via the transcellular route cannot be excluded.

In addition to studies on local effects, the possible occurrence of systemic effects 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 on blood pressure in the majority of animals. However, immediately after challenge some animals demonstrated a temporary drop in breathing frequency that returned to normal rates within 10 min. In respiratory allergy studies in guinea pigs, a drop in breathing frequency below 70% of the normal breathing frequency is referred to as an indication of severe respiratory effects (Botham et al., 1989). Although severe respiratory effects were observed in only a few animals (around 10–15%), this low incidence is in agreement with observations from food allergic patients, of whom only about 10% are reported to react with respiratory problems (Monteleone and Sherman, 1997). Also, the effects on systolic blood pressure, which occurred in approximately 40% of the challenged rats, were not dramatic and did not result in circulatory collapse. Two different patterns of blood pressure drop could be distinguished, in which some animals showed a continuous decrease and other animals a delayed decrease. Again, the rather low incidence of cardiovascular effects upon oral challenge of the rats is in accordance with human clinical experience. These observations indicate that in orally sensitized animals systemic effects can be induced upon an oral challenge.

4. Conclusion

It is evident that new approaches are needed to assess more appropriately the potential oral allergenicity of 'novel proteins'. The development of a predictive animal model is, in this context, often indicated to be of major importance in improving the currently used ILSI/IFBC decision tree. 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 physicochemical characteristics of known allergens, the ultimate proof of the presence/absence of sensitizing activity of the novel

proteins can be established in a predictive animal model.

The present paper summarizes studies focused on the development of an oral sensitization protocol, in which without the use of an adjuvant Brown Norway rats were sensitized to various allergenic food proteins (Knippels et al., 1998a,b, 1999a,b, 2000). The results clearly indicate that oral exposure of BN rats to food proteins may result in a significant IgE response to the proteins. In addition, when the BN rats were exposed to a mixture of proteins (HEW- and CM-protein extracts) specific antibody responses were elicited towards only some of the proteins whereas to the majority of proteins present in CM- and HEW-extract no specific IgE response was produced. The profile of allergens recognized by the immune system of the BN rat appeared comparable to that of allergens recognized by allergic-humans as measured by immunoblot techniques (Knippels et al., 2000).

After an oral OVA challenge in OVA sensitized BN rats effects were also shown on respiratory, circulatory and gastrointestinal functions. The possible occurrence of systemic effects upon an oral challenge was 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 a temporary decrease in breathing frequency or systolic blood pressure which is in accordance with human clinical experience (Knippels et al., 1999b). Following the studies on systemic effects, the occurrence of local effects upon an oral challenge was demonstrated by an increased gut permeability, as demonstrated by an increased uptake of the bystander protein β -LG 1.5 h after an oral challenge.

Although additional studies are needed to further validate the developed BN rat model, the results obtained to date indicate that the BN rat might be a useful and predictive animal model to study the potential oral allergenicity of 'novel' food proteins. Moreover, for studying more mechanistic aspects of food allergy, that might result in better possibilities with respect to prophylaxis and therapy, the BN rat model seems to be promising too.

References

- Atkinson, H.A.C., Johnson, I.T., Gee, J.M., 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.
- Bazin, H., Platteau, B., 1976. Production of circulating reaginic (IgE) antibodies by oral administration of ovalbumin to rats. *Immunology* 30, 679–684.
- Berin, M.C., Kiliaan, A.J., Yang, P.C., Groot, J.A., Taminiau, A.J.M., Perdue, M.H., 1997. Rapid transepithelial antigen transport in rat jejunum: impact of sensitization and the hypersensitivity reaction. *Gastroenterology* 113, 856–864.
- Bernhisel-Broadbent, J., Dintzis, M.H., Dintzis, R.Z., Sampson, H.A., 1994. Allergenicity and antigenicity of chicken egg ovomucoid (*Gal d III*) compared with ovalbumin (*Gal d I*) in children with egg allergy and in mice. *J. Allergy Clin. Immunol.* 93, 1047–1059.
- Botham, P.A., Ratray, N.J., Woodcock, D.R., Walsh, S.T., Hext, P.M., 1989. The induction of respiratory allergy in guinea pigs following intradermal injection of trimellitic anhydride: a comparison with the response to 2,4-dinitrochlorobenzene. *Toxicol. Lett.* 47, 25–39.
- Bruijnzeel-Koomen, C., Ortolani, C., Aas, K., Bindeslev-Jensen, C., Bjorksten, B., Moneret-Vautrin, D., Wutrich, B., 1995. Adverse reactions to food. *Allergy* 50, 623–635.
- Challacombe, S.J., Tomasi, T.B., 1980. Systemic tolerance and secretory immunity after oral immunisation. *J. Exp. Med.* 152, 1459–1472.
- Crowe, S.E., Perdue, M.H., 1992. Gastrointestinal food hypersensitivity; basic mechanisms of pathophysiology. *Gastroenterology* 103, 1075–1095.
- Dearman, R.J., Caddick, H., Basketter, D.A., Kimber, I., 2000. Divergent antibody isotope responses induced in mice by systemic exposure to proteins: a comparison of ovalbumin with bovine serum albumin. *Food Chem. Toxicol.* 38, 351–360.
- Devey, M.E., Anderson, K.J., Coombs, R.R.A., 1976. The modified anaphylaxis hypothesis for cot death. Anaphylactic sensitization in guinea pigs fed cow's milk. *Clin. Exp. Immunol.* 26, 542–548.
- Ebbehoj, K., Dahl, A.M., Frokiaer, H., Norgaard, A., Poulsen, L.K., Barkholt, V., 1995. Purification of egg white allergens. *Allergy* 50, 133–141.
- Ermel, R.W., Kock, M., Griffey, S.M., Reinhart, G.A., Frick, O.L., 1997. The atopic dog: a model for food allergy. *Lab. Anim. Sci.* 47, 40–49.
- Heyman, M., Darmon, N., Dupont, C., Dugas, B., Hirribaren, A., Blaton, M.-A., Desjeux, J.-F., 1994. Mononuclear cells from infants allergic to cow's milk secrete tumor necrosis factor, altering intestinal function. *Gastroenterology* 106, 1514–1523.
- Hilton, J., Dearman, R.J., Basketter, D.A., Kimber, I., 1994. Serological responses induced in mice by immunogenic proteins and by protein respiratory allergens. *Toxicol. Lett.* 73, 43–53.

- Hilton, J., Dearman, R.J., Satter, N., Basketter, D.A., Kimber, I., 1997. Characteristics of antibody responses induced in mice by protein allergens. *Food Chem. Toxicol.* 35, 1209–1218.
- Ito, K., Ohara, K.I., Murosaki, S., Nishimura, H., Shimokata, T., Torii, S., Matsuda, T., Yoshikai, Y., 1997. Murine model of IgE production with a predominant Th2-response by feeding protein antigen without adjuvants. *Eur. J. Immunol.* 27, 3427–3437.
- Kanwar, S., Wallave, J.L., Befus, D., Kubes, P., 1994. Nitric oxide synthesis inhibition increases epithelial permeability via mast cells. *Am. J. Physiol. (Gastrointest. Liver Physiol.)* 266, G222–G229.
- Knippels, L.M.J., Penninks, A.H., Spanhaak, S., Houben, G.F., 1998a. Oral sensitization to food proteins: a Brown Norway rat model. *Clin. Exp. Allergy* 28, 368–375.
- Knippels, L.M.J., Penninks, A.H., Houben, G., 1998b. Continued expression of anti soy-protein antibodies in rats bred on a soy-protein free diet; the importance of dietary control in oral sensitization research. *J. Allergy Clin. Immunol.* 101, 815–820.
- Knippels, L.M.J., Penninks, A.H., van Meeteren, M., Houben, G.F., 1999a. Humoral and cellular immune responses in different rat strains upon oral exposure to ovalbumin. *Food Chem. Toxicol.* 37, 881–888.
- Knippels, L.M.J., Penninks, A.H., Smit, J.J., Houben, G.F., 1999b. 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, L.M.J., Felijs, A.A., van der Kleij, H.P.M., Penninks, A.H., Koppelman, S.J., Houben, G.F., 2000. Comparison of antibody responses to hen's egg and cow's milk-proteins in orally sensitized rats and human patients. *Allergy* 55, 251–258.
- Li, X., Schofield, B.H., Huang, C.K., Kleiner, G.I., Sampson, H.A., 1999. A murine model for IgE-mediated cow's milk hypersensitivity. *J. Allergy Clin. Immunol.* 103, 206–214.
- Li, X., Serebrisky, D., Lee, S.J., Huang, C.K., Bardina, L., Schofield, B.H., Stanley, J.S., Burks, A.W., Bannon, G.A., Sampson, H.A., 2000. A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. *J. Allergy Clin. Immunol.* 106, 150–158.
- Metcalfe, D.D., Astwood, J.D., Townsend, R., Sampson, H.A., Taylor, S.L., Fuchs, R.L., 1996. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Crit. Rev. Fd. Sci. Nutr. (S)* 36, 165–186.
- Monteleone, C.A., Sherman, A.R., 1997. Nutrition and asthma. *Arch. Intern. Med.* 157, 23–24.
- Mowat, A.M., 1987. The regulation of the immune responses to dietary protein antigens. *Immunol. Today* 8, 93–98.
- Ovary, Z., 1964. Passive cutaneous anaphylaxis. *Immunol. Methods. A Symposium.* Ackroyd, J.F. (ed.) Blackwell (Oxford), 259–283.
- Pauwels, R., Bazin, H., Platteau, B., van der Straeten, M., 1979. The effect of age on IgE production in rats. *Immunology* 36, 145–159.
- Piacentini, G.L., Bertolini, A., Spezia, E., Piscione, T., Boner, A.L., 1994. Ability of a new infant formula prepared from partially hydrolyzed whey to induce anaphylactic sensitization; evaluation in a guinea pig model. *Allergy* 49, 361–364.
- Scudamore, C.L., Thornton, E.M., McMillan, L., Newlands, G.F.J., Miller, H.R.P., 1995. Release of the mucosal mast cell granule chymase, rat mast cell protease II, during anaphylaxis is associated with the rapid development of paracellular permeability to macromolecules in rat jejunum. *J. Exp. Med.* 182, 1871–1881.
- Steinmann, J., Wottge, H.U., 1990. Immunogenicity testing of food proteins: in vivo and in vitro trials in rats. *Int. Arch. Allergy Appl. Immunol.* 91, 62–70.
- Strobel, S., Ferguson, A., 1984. Immune responses to fed protein antigens in mice. Systemic tolerance or priming is related to age at which antigen is first encountered. *Pediatr. Res.* 18, 588–594.
- Strobel, S., Ferguson, A., 1986. Modulation of intestinal and systemic immune responses to a fed protein antigen in mice. *Gut* 27, 829–837.
- Taylor, S.L., Leher, S.B., 1996. Principles and characteristics of food allergens. *Crit. Rev. Fd. Sci. Nutr. (S)* 36, 91–118.
- Turner, M.W., Barnett, G.E., Strobel, S., 1990. Mucosal mast cell activation patterns in the rat following repeated feeding of antigen. *Clin. Exp. Allergy* 20, 421–427.
- Wal, J.M., 1999. Assessment of allergic potential of (novel) foods. *Nahrung* 43, 168.