

Assessment of Protein Allergenicity

Studies in Brown Norway Rats

LÉON M.J. KNIPPELS AND ANDRÉ H. PENNINKS

Experimental Immunology, Department of Target Organ Toxicology, TNO Nutrition and Food Research, 3700 AJ Zeist, The Netherlands

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 allergic sensitizing ability of food proteins. Therefore, there is a growing interest in the development of animal models. This paper summarizes the results of a promising food allergy model developed in Brown Norway (BN) rats. The results demonstrate that BN rats can be sensitized via the relevant oral route of exposure. Daily gavage dosing of the animals with several food proteins, without the use of adjuvants, resulted in significant antigen-specific IgE responses. In addition, the profile of allergens recognized by the immune system of the BN rat, appeared comparable to the profile of allergens recognized by allergic humans. Besides oral sensitization, local and systemic immune-mediated effects, such as increased gastrointestinal permeability, decreased breathing frequency, and decreased blood pressure, could also be observed in the sensitized animals after an oral challenge. All together, these observations suggest that this BN rat model might provide a suitable animal model to study the allergenicity of food proteins in humans.

KEYWORDS: protein allergenicity; Brown Norway rats; genetically engineered crops; animal models; passive, cutaneous anaphylaxis testing; IgE

INTRODUCTION

With a growing interest in the development of genetically engineered food crops, there is a need for appropriate approaches to safety assessment.^{1,2} 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.³ Until recently, one of the best known allergy assessment protocols was the careful

Address for correspondence: Léon M.J. Knippels, Ph.D., Study Director Experimental Immunology, Department of Target Organ Toxicology, TNO Nutrition and Food Research, P.O. Box 360, 3700 AJ Zeist, The Netherlands. Voice: +31 30 694 43 78; fax: +31 30 696 02 64. knippels@voeding.tno.nl

Ann. N.Y. Acad. Sci. 964: 151–161 (2002). © 2002 New York Academy of Sciences.

stepwise process using a decision tree strategy as suggested by the International Life Sciences Institute (ILSI) Allergy and Immunology Institute and the International Food Biotechnology Council (IFBC).⁴ A hierarchical testing strategy was proposed, which for genes derived from nonallergenic sources includes o.a. 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.^{1,4,5} Unfortunately, these tests are not conclusive with respect to the potential allergenicity of the protein involved. In case negative predictions are observed for all recommended tests, there is a low probability that the protein is a significant food allergen, but still full assurance cannot be given with respect to the sensitizing potential of the protein studied. Especially if a positive prediction is observed for one of the listed parameters, further testing is warranted that should in particular be focused on the prediction of the sensitizing potential of the novel protein. Recently, the FAO/WHO has proposed a revised decision tree strategy⁶ for allergenicity testing, in which o.a. additional serum screening and animal models are introduced. Despite the fact that validated animal models from which the results can be extrapolated to humans are not yet available, new developments are in progress. Because of the complexity of the immune system and the different factors known to influence sensitization in susceptible individuals, it is not expected that this potential allergenicity of (novel) proteins 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(s). 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 its potency relative to well-known allergens.

In our laboratory we have been working on the development of an oral Brown Norway (BN) rat food allergy model. We have chosen the BN rat because 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. The animals were successfully sensitized using a daily oral gavage dosing protocol.⁷ To study whether the BN rat is indeed the preferable strain of rats, we performed comparative sensitization studies 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, which confirmed the choice of the BN rat for oral sensitization studies.⁸ 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 allergic patients to these allergens and was found to be directed to the same proteins in these allergenic foods.⁹ One important factor that may have negatively affected the results of many oral sen-

sitization studies is the unscheduled dietary pre-exposure of test animals or their parental generation to the antigen under investigation. As shown in our 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.¹⁰ Therefore, when oral sensitization studies with proteins are performed, one has to ensure that at least two generations of animals are bred on a diet free of the antigen under investigation to get immunologically naïve animals. The BN rat food allergy model was further characterized by studying immune-mediated effects on an oral challenge showing effects on breathing frequency, gastrointestinal permeability, and blood pressure.¹¹ All together, these observations suggest that the BN rat model might provide a suitable animal model to study the allergenicity of food proteins, although further validation of the model is needed.

MATERIALS AND METHODS

Rats and Protein Extracts

For the sensitization studies, young male BN rats were obtained from Charles River (Sulzfeld, Germany) that were 4–6 weeks old at study initiation. During the experiment and for at least 10 days before the 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 hr 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. Because hen's egg-white (HEW) proteins, purified ovalbumin (OVA) from HEW, and cow's milk (CM) proteins 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, England). To ensure the use of immunologically naïve animals with respect to the antigens under investigation, pre-study blood samples were always tested for HEW-protein- and CM-protein-specific antibodies.

The total 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.*¹² 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 microcentrifuged for 15 min at $17,000 \times g$ and 4°C and subsequently filter-sterilized through 0.2- μm filters (Costar Scientific Corporation, Cambridge, MA). The protein content of the extract was determined with a BCA protein assay (Pierce, Rockford, IL) according to the manufac-

turer'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%).

Sensitization Protocol

Oral sensitization of the animals was achieved by gavage dosing, using a 18-gauge stainless steel animal feeding needle to feed OVA, HEW proteins, or CM proteins (1 mg or 10 mg protein/ml tap water; 1 ml/animal) over a 6-week period, without the use of an adjuvant. At weekly intervals, blood samples were obtained from the orbital plexus under light CO₂ anesthesia or by exsanguination from the abdominal aorta at sacrifice. After coagulation for 1 hr at room temperature, the blood samples were centrifuged (Heraeus Mini-fuge T, Osterode, Germany) for 20 min at 2000 × *g* and 4°C to obtain sera. The sera were stored at -20°C until analyses for anti-OVA and 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.

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-well 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: >98%) or with a 75-µg/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 µl/well phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA; Sigma Chemicals Co., St. Louis, MO) and 0.02% Tween 20 (PBS/BSA-Tween 20). After a 1-hr 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 hr at 37°C. After washing, 100 µl/well peroxidase-conjugated goat anti-rat IgG (H+L) (Zymed, San Francisco, CA, diluted 1:500) in PBS/BSA-Tween 20 was added. After incubation for 1 hr at 37°C, the plates were washed again, and an enzyme substrate solution of 3,3',5,5'-tetramethylbenzidine (TMB; Sigma Chemicals Co., St. Louis, MO, 100 µl/well; 6 mg/ml DMSO) was added. The plates were developed at room temperature for 5 to 15 min. Finally, 100 µl/well of 2N H₂SO₄ was added. Optical densities were read spectrophotometrically at 450 nm with an ELISA

plate reader (Microplate Reader, Bio-Rad Laboratories, Richmond, VA). A preserum pool was used as 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 serum. 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-well plate.

For the detection of OVA-specific IgE, 96-well microtiter plates were coated overnight at 4°C with 100 µl/well mouse anti-rat IgE (MARE-1, Zymed, San Francisco, CA) at a concentration of 1.5 µg/ml in carbonate buffer, pH 9.6. The plates were washed, and 100 µl/well of PBS/BSA-Tween 20 was added. After incubation for 1 hr at 37°C, the plates were washed, and diluted rat serum samples were added and incubated for 2 hr at 37°C. The plates were washed, and subsequently 100 µl/well of a 1 µg/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 labeled OVA was separated on a sephadex G-25 column (Boehringer, Mannheim, Germany), and labeling efficiency was determined spectrophotometrically at 280 nm. Incubation with OVA-DIG was performed for 1 hr at 37°C. After washing, 100 µl/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 hr 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.

Measurement of Reagenic Antibodies by Passive Cutaneous Anaphylaxis

For passive cutaneous anaphylaxis (PCA) testing, the method as described previously by Ovary¹³ was used. Naive (untreated) BN rats were shaven on the back and flanks and injected intradermally with 0.1 ml of the test serum in serial dilutions, followed 64 hr later with an intravenous injection of 1 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 reaginic titer was read as the reciprocal of the furthest dilution giving a colored spot of at least 5 mm in diameter. Positive and negative control sera as used in the ELISA's were assayed simultaneously with the test serum on each animal used for the PCA tests.

Measurement of Gut Permeability

In order to study possible changes in gut permeability in sensitized rats, the uptake of a bystander protein, namely, β -lactoglobulin, was followed in time after an oral challenge. Therefore, OVA-sensitized and control animals were orally challenged 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 intragastric dose of β -lactoglobulin (β -LG, 1 ml of a 100 mg/ml solution in tap water; obtained from Sigma Chemicals Co., St. Louis, MO, purity 90%). At 0.5, 1, 2, 3 and 5 hr after the β -LG administration, blood samples were collected from the orbital plexus under light CO₂ anesthesia or at sacrifice 8 hr 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.¹¹

Measurement of Blood Pressure

In order to determine the possible changes in blood pressure, rats were trained, during the sensitization period to get them 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. Before 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 animals and the control animals were orally challenged 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 during a period of 7 hr.

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 on one side and thorax and abdomen on 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 that sensed changes created by inhalation and expiration and transmitted amplified signals to a polygraph recorder, thus allowing determination of respira-

tory 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 orally challenged 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. Before challenge, the respiratory function was measured constantly for 15 min and, immediately upon an oral challenge with OVA, constantly during the first 10 min and thereafter for periods of 30 sec once every 5 min for a total period of 6 hours.

RESULTS

Antibody Responses to OVA/HEW/CM

Daily intragastric 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 our studies exceeded 80%, as measured by ELISA and PCA. Optimal specific IgE antibody responses were observed around days 28–35. Occasionally, however, no OVA-specific IgE responses were induced upon daily gavage dosing with OVA in our studies. Using less frequent administration regimes of 1 mg OVA by gavage did not, in general, induce specific IgG or specific IgE antibody responses. Upon *ad libitum* exposure, only OVA-specific IgG but no OVA-specific IgE was produced.⁷

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.⁹ Moreover, antibodies present in sera of rats orally sensitized to HEW or CM and in sera of patients allergic 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 orally exposed to HEW proteins recognized a whole scale 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 orally exposed to HEW proteins recognized the same proteins, namely, ovotransferrin, ovalbumin, ovomucoid, and to a lesser extent lysozyme, which are claimed to be the major allergens for hen's egg-allergic humans.¹⁴ 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 orally sensitized to CM proteins

and antibodies present in sera from milk-allergic patients was compared. Although cow's milk contains more than 30 proteins, the induced antibodies were mainly directed against β -lactoglobulin 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 intragastric 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 intraperitoneally sensitized with CM, which produced a profile of IgE antibodies to milk proteins similar to that observed in humans.¹⁵ 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. 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 developed rat model, additional studies were performed in OVA-sensitized rats to study local and systemic immune-mediated effects upon an oral challenge with OVA.⁹

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 to effects on the gastrointestinal tract physiology or on the respiratory and/or cardiovascular system, to skin effects.¹⁶ For validation purposes in the BN rat model, oral challenge reactions should also be studied to show whether clinical reactions can be induced and which organs are involved. Therefore, the animals previously sensitized with OVA were orally challenged 10 days after the oral sensitization period by intragastric intubation with doses of OVA varying between 10 and 100 mg. Subsequently, effects on gastrointestinal permeability, blood pressure, and breathing frequency were investigated.¹¹

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 min later, the amount of β -LG in the sera of previously sensitized

rats was significantly higher than in sera of nonsensitized 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.^{17,19} The release of mediators in anaphylactic reactions such as histamine, platelet-activating factor, prostaglandins, leukotrienes, and some newly formed cytokines have also been shown to alter mucosal function in experimental models.^{19,20} Whether the increased macromolecular passage is mainly due to transcellular or paracellular transport is still not clear. Scudamore *et al.*,²¹ however, 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 our 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 increased permeability via the transcellular route cannot be excluded.

In addition to studies on local effects, the possible occurrence of systemic effects 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 animals. Nevertheless, 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.²² Although we only observed severe respiratory effects in a few animals (around 10–15% of the animals), this low incidence is in agreement with observations from food-allergic patients, of whom only about 10% are reported to react with respiratory problems.²³ The effects on systolic blood pressure, which occurred in approximately 40% of the challenged rats, were also not dramatic and did not result in a 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 the human clinical practice. These observations indicate that in orally sensitized animals, systemic effects can be induced upon an oral challenge.

CONCLUSIONS

In this paper studies are summarized that focus 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.^{7–11} 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 or CM protein extracts), specific antibody responses were only elicited towards several proteins, whereas no specific IgE response to the majority of proteins present in CM and HEW extracts was produced. The profile of allergens recognized by the immune system of the BN rat appeared comparable to the profile of allergens recognized by allergic humans as measured by immunoblot techniques.⁹ After an oral ovalbumin (OVA) challenge in the OVA-sensitized BN rats, effects were also shown on respiratory, circulatory, and gastrointestinal functions. 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. Some animals, however, demonstrated a temporary decrease in breathing frequency or systolic blood pressure, which is in accordance with human clinical experience.¹¹ 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 -lactoglobulin 1.5 hr after an oral challenge.

The available evidence suggests that the BN rat may provide an appropriate model for the identification and characterization of specific proteins' allergens upon exposure via the relevant oral route. Although additional studies are needed to investigate whether the model is able to distinguish between strong, weak, and nonallergenic proteins, the results obtained up until now indicate that the BN rat might be a useful and predictive animal model in which to study the potential oral allergenicity of "novel" food proteins.

REFERENCES

1. ASTWOOD, J.D., J.N. LEACH & R.L. FUCHS. 1996. Stability of food allergens to digestion in vitro. *Nat. Biotechnol.* **14**: 1269–1273.
2. TAYLOR, S.L. 1997. Food from genetically modified organisms and potential for food allergy. *Environ. Toxicol. Pharmacol.* **4**: 121–126.
3. WAL, J.M. 1999. Assessment of allergic potential of (novel) foods. *Nahrung* **43(3)**: 168–174.
4. METCALFE, D.D., J.D. ASTWOOD, R. TOWNSEND, *et al.* 1996. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Crit. Rev. Food Sci. Nutr.* **36(S)**: 165–186.
5. GENDEL, S.M. 1998. Sequence databases for assessing the potential allergenicity of proteins used in transgenic foods. *Adv. Food Nutr. Res.* **42**: 63–92.
6. U.N. FOOD AND AGRICULTURAL ORGANIZATION/WORLD HEALTH ORGANIZATION. 2001. FAO/WHO evaluation of allergenicity of genetically modified foods: expert consultation. Food and Agricultural Organization of the United Nations. Rome, Italy. 22–25 January 2001, pp. 1–26.

7. KNIPPELS, L.M.J., A.H. PENNINKS, S. SPANHAAK & G.F. HOUBEN. 1998. Oral sensitization to food proteins: a Brown Norway rat model. *Clin. Exp. Allergy* **28**: 368–375.
8. KNIPPELS, L.M.J., A.H. PENNINKS, M. VAN MEETEREN & G.F. HOUBEN. 1999. Humoral and cellular immune responses in different rat strains upon oral exposure to ovalbumin. *Food Chem. Toxicol.* **37**: 881–888.
9. KNIPPELS, L.M.J., A.A. FELIUS, H.P.M. VAN DER KLEIJ, *et al.* 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.
10. KNIPPELS, L.M.J., A.H. PENNINKS & G. HOUBEN. 1998. 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.
11. KNIPPELS, L.M.J., A.H. PENNINKS, J.J. SMIT & G.F. HOUBEN. 1999. 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.
12. BERNHISEL-BROADBENT, J., M.H. DINTZIS, R.Z. DINTZIS & H.A. SAMPSON. 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.
13. OVARY, Z. 1964. Passive cutaneous anaphylaxis. *Immunol. Methods*: 259–283.
14. EBBEHOJ, K., A.M. DAHL, H. FROKIAER, *et al.* 1995. Purification of egg white allergens. *Allergy* **50**: 133–141.
15. ATKINSON, H.A.C., I.T. JOHNSON, J.M. GEE, *et al.* 1996. Brown Norway rat model of food allergy: effect of plant components on the development of oral sensitization. *Food Chem. Toxicol.* **34**: 27–32.
16. BRUINZEEL-KOOMEN, C., C. ORTOLANI, K. AAS, *et al.* 1995. Adverse reactions to food. *Allergy* **50**: 623–635.
17. CROWE, S.E. & M.H. PERDUE. 1992. Gastrointestinal food hypersensitivity: basic mechanisms of pathophysiology. *Gastroenterology* **103**: 1075–1095.
18. BERIN, M.C., A.J. KILIAAN, P.C. YANG, *et al.* 1997. Rapid transepithelial antigen transport in rat jejunum: impact of sensitization and the hypersensitivity reaction. *Gastroenterology* **113**: 856–864.
19. HEYMAN, M., N. DARMON, C. DUPONT, *et al.* 1994. Mononuclear cells from infants allergic to cow's milk secrete tumor necrosis factor, altering intestinal function. *Gastroenterology* **106**: 1514–1523.
20. KANWAR, S., J.L. WALLAVE, D. BEFUS & P. KUBES. 1994. Nitric oxide synthesis inhibition increases epithelial permeability via mast cells. *Am. J. Physiol. (Gastrointest. Liver Physiol.)* **266**: G222–229.
21. SCUDAMORE, C.L., E.M. THORNTON, L. McMILLAN, *et al.* 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.
22. BOTHAM, P.A., N.J. RATTRAY, D.R. WOODCOCK, *et al.* 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.
23. Monteleone, C.A. & A.R. Sherman. 1997. Nutrition and asthma. *Arch. Intern. Med.* **157**: 23–24.

