

An Oral Sensitization Model in Brown Norway Rats to Screen for Potential Allergenicity of Food Proteins

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We developed an oral sensitization protocol for food proteins for the rat. Young Brown Norway (BN) rats were exposed to 1 mg ovalbumin (OVA) by daily gavage dosing for 42 days without the use of an adjuvant. OVA-specific IgE and IgG responses were determined by ELISA. On an oral challenge with OVA some clinical symptoms of food allergy-like effects on the respiratory system, blood pressure, and permeability of the gastrointestinal barrier were studied. In addition, BN rats were orally exposed to a total hen egg white protein (HEW) extract and cow's milk (CM) and the specificities of induced antibody responses were compared with the specificities of antibodies in sera from egg- and milk-allergic patients using immunoblotting. Animals orally exposed to the allergens developed specific IgE and IgG antibodies which recognized the same proteins compared with antibodies from egg- or CM-allergic patients. Among the various clinical symptoms of food allergy, gut permeability was increased after an oral challenge. In addition, some animals demonstrated a temporary decrease in breathing frequency or systolic blood pressure. The results obtained show that the Brown Norway rat is a suitable animal model for inducing specific IgG and IgE responses on daily intragastric dosing of OVA without the use of an adjuvant. Moreover, local immune-mediated effects on oral challenge are observed. The observation that enterally exposed BN rats and food-allergic patients demonstrate antibody responses to a comparable selection of proteins on exposure to different protein mixtures (HEW and CM) further supports the suitability of the BN rat as an animal model for food allergy research and for the study of the allergenicity of (novel) food proteins. © 1999 Academic Press

Food allergy is a problem of growing interest for several reasons. First, there are indications that an

increasing number of sensitized individuals suffer from the clinical features on a renewed contact with the offending food. Second, it is also anticipated that for the fast developments in biotechnology, which will result in the introduction of new proteins and new biological varieties for application in food, the potential allergenicity will pose a major concern in their safety assessment. The assessment strategy currently used seems to be promising for the testing of foods containing newly introduced gene products derived from common or less common allergenic sources. However, the possible transfer of a gene coding for an allergen from a source with no history of allergenicity is extremely difficult to predict with the current available tools.

Validated animal models to study food allergy and the allergenicity of food proteins are therefore urgently needed. This article presents information concerning a new rat model for allergenicity testing. Based on the results obtained up to now it is likely to become a valuable tool, not only for improvement of our knowledge on food allergy in general, but also for the assessment of the allergenic potential of genetically improved foods.

Several attempts have been made to develop animal models for food allergy research, mainly in mouse, guinea pig, and rat. Many of these studies have been conducted using parenteral sensitization and enteral challenges (1–4). Although effects on oral challenge in these models of IgE mediated hypersensitivity were successfully investigated, the natural route of feeding during the sensitization period was not taken into account. For the rat, oral sensitization to food proteins administered through the diet or by intragastric dosing was also reported (5–7). Although sensitization to orally administered food proteins can be induced in rats, tolerance induction may also occur (8). Neverthe-

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less, since the rat is commonly used in routine toxicity testing, knowledge on the oral sensitizing properties of food proteins in the rat would enable the evaluation of such properties in a perspective to the total of information on the potential effects of a product on the health state.

The development of IgE-mediated allergies, including food allergy, is more common in atopic humans who have a genetic predisposition to react with an elevated production of IgE antibodies to generally harmless substances (9). The Brown Norway (BN) rat is a high-immunoglobulin (particularly IgE) responder strain (10) and thus, to a certain degree, resembles atopic humans in their (genetic) predisposition to react with an overproduction of IgE to antigens. Due to this preferential response the BN rat was chosen for the development of an oral sensitization model, in which ovalbumin, a well-defined chicken egg white allergen, was used as a model allergen (11).

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 get immunologically naive animals. It has been shown that exposure of the parental generation to the antigen under investigation can influence the outcome of sensitization studies with the offspring (12). The BN rats used in our studies met this condition.

DESCRIPTION OF METHODS

Materials

In the oral sensitization studies, three different antigen sources were used. A total hen egg white (HEW) protein extract was prepared essentially according to the method of Bernhisel-Broadbent *et al.* (13). Hen eggs (free-range egg) were obtained from Albert Heijn, Zaandam, The Netherlands. 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 2500g and 4°C. The tubes were decanted and the supernatants were microcentrifuged for 15 min at 17,000g and 4°C and subsequently filter-sterilized through 0.2- μ m filters (Costar Scientific Corp., Cambridge, MA). The protein content of the extract was determined with a BCA protein assay (Pierce, Rockford, IL) according to the manufacturer's instructions. The total protein content of the extract was 24 mg protein/ml saline and the total protein yield was approximately 6.5 g. The two other antigen sources used were ovalbumin (OVA; Fluka Chemie, Buchs, Switzerland, purity: 70%) and skimmed cow's milk (CM; Albert Heijn, Zaandam, The Netherlands, 4 g protein/100 ml milk).

Oral Sensitization Protocol

Young male BN rats, 4–6 weeks old at study initiation, were obtained from Charles River (Sulzfeld, Germany). 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% during the experiment and for at least 10 days prior to study initiation. The animals were housed in stainless-steel wire cages in groups of four or five and had free access to food and tap water. 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]. Prestudy blood samples were always tested for HEW protein- and CM protein-specific antibodies to ensure the use of immunologically naive animals with respect to the antigens under investigation.

The animals were exposed to OVA, HEW proteins, or CM proteins by daily gavage dosing, using an 18-gauge stainless-steel animal feeding needle (1 mg protein/ml tap water; 1 ml/animal) for 6 weeks, without the use of an adjuvant. Less frequent intragastric intubation with OVA or exposure to OVA via the drinking water did not result in sensitization of the animals (11). Blood samples were obtained from the orbital plexus under light CO₂ anesthesia at weekly intervals or by exsanguination from the abdominal aorta at sacrifice. After coagulation for 1 h at room temperature, the blood samples were centrifuged (Heraeus Minifuge T, Osterode, Germany) for 20 min at 2000g 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.

Assays for Anti-OVA Antibodies

Serum antibodies specific for OVA, HEW proteins, or CM-proteins were measured by ELISA. 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 μ l/well of a 5 μ g/ml solution of OVA (Serva, Feinbiochemica, Heidelberg/New York, purity: >98%) or CM proteins or with a 75 μ g/ml solution of HEW 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 μ l/well phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA; Sigma Chemical Co., 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 μ l/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 h at 37°C, the plates were washed again and an enzyme substrate solution of 3,3',5,5'-tetramethylbenzidine (TMB; Sigma Chemical Co.; St. Louis, MO, 100 μ l/well, 6 mg/ml DMSO) was added. The plates were developed at room temperature for 5 to 15 min. Finally, 100 μ l/well of 2 N H₂SO₄ was added. Optical densities were read spectrophotometrically at 450 nm with an ELISA plate reader (Microplate Reader, Bio-Rad Laboratories, Richmond, CA). A pre-serum 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 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-well plate.

For the detection of OVA-specific IgE, 96-well microtiter plates were coated overnight at 4°C with 100 μ l/well mouse anti-rat IgE (MARE-1, Zymed) at a concentration of 1.5 μ g/ml in carbonate buffer, pH 9.6. The plates were washed and 100 μ 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 μ l/well of a 1 μ 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 labeled OVA was separated on a Sephadex G-25 column (Boehringer) and labeling efficiency was determined spectrophotometrically at 280 nm. Incubation with OVA-DIG was performed for 1 h at 37°C. After washing, 100 μ l/well peroxidase-conjugated sheep anti-DIG Fab fragments (Boehringer) 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.

Passive Cutaneous Anaphylaxis

PCA was tested essentially as described previously by Ovary *et al.* (14). 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 h 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 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.

Challenge Effects

In a newly developed animal model in addition to oral sensitization, also the challenge reactions (clinical reactions) must be induced orally and show the involvement of the various organs also affected in patients. 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. On an oral challenge effects on blood pressure, gastrointestinal permeability, and breathing frequency were investigated (15).

Determination of Respiratory Function

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. Respiratory frequency was assessed using a plethysmograph with a separate head and body chamber and matched pressure transducers (Fig. 1). Each plethysmograph was provided with a pressure transducer that sensed changes created by inspiration and expiration and transmitted amplified signals to a polygraph recorder, so allowing determination of respiratory frequency and pattern. Rats were restrained in modified Batelle tubes (Batelle, Geneve, Switzerland) with a water-wetted silicone diaphragm to give an airtight seal between head and neck at the one side and thorax and abdomen at the other. The tube was placed in the body chamber with the open end of the tube fitting into the front chamber. Breathing frequencies were determined by means of recording the pressure signal in the volume-calibrated body chamber. Prior to challenge, the respiratory function was measured constantly for 15 min and immediately on an oral challenge with OVA constantly during the first 10 min and thereafter for periods of 30 s once every 5 min for a total period of 6 h.

Determination of Blood Pressure

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. During the sensitization period, the rats were trained to get used to the measurement equipment 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 placed 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). Blood pressures were recorded at intervals during a period of 7 h.

Determination of Gut Permeability

To determine possible changes in gut permeability, the uptake of a bystander protein was determined following challenge. Animals sensitized with OVA 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. 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; purity: 90%) 30 min after the oral challenge with OVA. Blood samples were collected from the orbital plexus under light CO₂ anaesthesia at 0.5, 1, 2, 3, and 5 h after the β -LG administration or by exsanguination from the abdominal aorta at sacrifice 8 h after the β -LG administration. Sera were prepared and stored as described and used for the quantification of β -LG by ELISA (15).

CONCLUDING REMARKS

New approaches that are appropriate for the assessment of the potential oral allergenicity of “novel

proteins” are needed. In this context, the development of a predictive animal model is often indicated as urgently needed. Especially for those products with an unknown history of allergenicity or those that show one or more physio/chemical characteristics of known allergens, the ultimate proof for presence/absence of sensitizing activity of the novel proteins can be studied in a predictive animal model. In the present paper an oral sensitization protocol is described, without the use of an adjuvant, to sensitize Brown Norway rats to food proteins. The most significant results are summarized in Table 1. From these results it appears that oral exposure to food proteins results in a significant IgE response to the proteins. In addition, exposure to a mixture of proteins (cow's milk or hen egg white protein extract) resulted in specific antibody responses toward several proteins while the majority of proteins present in CM and HEW extract did not elicit a specific IgE response in the BN rat. 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 immunoblotting (15). Besides oral sensitization we also showed effects after an oral challenge in the sensitized animals. We showed respiratory, circulatory, and gastrointestinal effects on challenge of ovalbumin-sensitized BN rats. On an oral OVA challenge, gut permeability was increased as demonstrated by increased uptake of a bystander protein, β -lactoglobulin, 1.5 h after an oral challenge. Following the studies on local effects, the possible occurrence of systemic effects on an oral challenge were

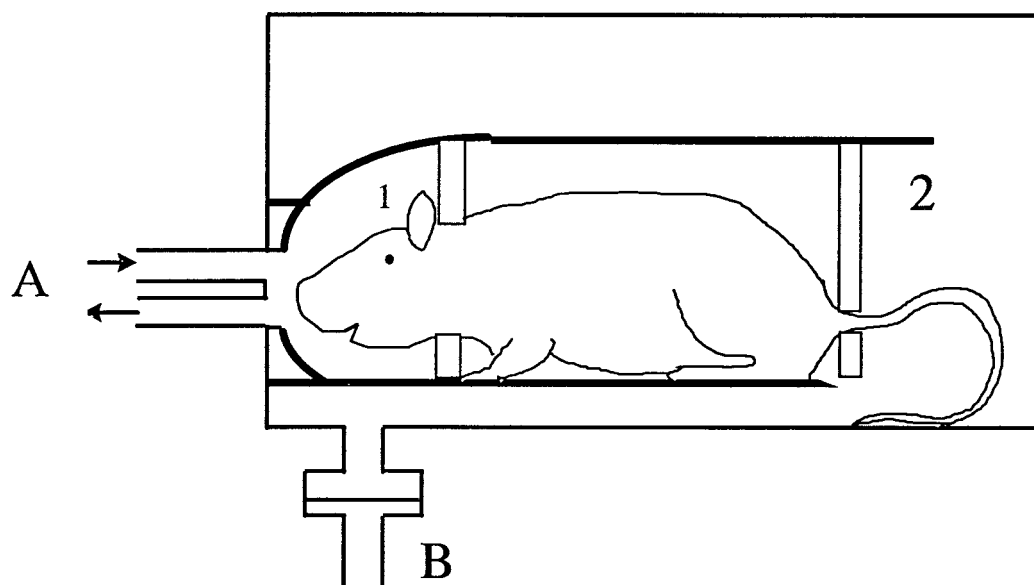


FIG. 1. Schematic presentation of a two-chambered plethysmograph: (1) head section, (2) body section, (A) fresh air supply, (B) pressure device.

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 the human clinical practice (15).

Although several additional studies are needed to further validate the developed BN rat model, the results obtained until now indicate that the BN rat seems to be a useful animal model to study oral sensitization to food proteins as well as immune-mediated effects on oral challenge with food proteins. In addition, the model seems promising for studying mechanistic aspects of food allergy.

TABLE 1

Characteristics of Brown Norway Rat Model

Oral sensitization to food proteins, without the use of an adjuvant, results in a significant IgE response to the food proteins.
IgE responses to hen egg white and cow milk proteins in the BN rat are directed to the same proteins as compared with IgE antibodies present in sera from egg- and milk-allergic patients.
After an oral challenge, in previously sensitized animals, local and systemic immune mediated effects are observed: increase in permeability of the gastrointestinal tract, decrease in breathing frequency, and decrease in blood pressure.

REFERENCES

1. Byars, N. E., and Ferraresi, R. W. (1976) *Clin. Exp. Immunol.* **24**, 352–356.

2. Freier, S., Eran, M., and Goldstein, R. (1985) *Pediatr. Res.* **19**, 456–459.

3. Turner, M. W., Barnett, G. E., and Strobel, S. (1990) *Clin. Exp. Allergy* **20**, 421–427.

4. Curtis, G. H., Patrick, M. K., Catto-Smith, A. G., and Gall, D. G. (1990) *Gastroenterology* **98**, 1558–1566.

5. Bazin, H., and Platteau, B. (1976) *Immunology* **30**, 679–684.

6. Jarrett, E. E. E., Haig, D. M., McDougall, W., and McNulty, E. (1976) *Immunology* **30**, 671–677.

7. Atkinson, H. A. C., Johnson, I. T., Gee, J. M., Grigoriadou, F., and Miller, K. (1996) *Food Chem. Toxicol.* **34**, 27–32.

8. Steinmann, J., Wottge, H. U., and Müller-Ruchholtz. (1990) *Int. Arch. Allergy Appl. Immunol.* **91**, 62–65.

9. Bruijnzeel-Koomen, C., Ortolani, C., Aas, K., Bindeslev-Jensen, C., Björkstén, B., Moneret-Vautrin, D., and Wütrich, B. (1995) *Allergy* **50**, 623–635.

10. Holt, P., and Turner, K. J. (1985) *Int. Arch. Allergy Appl. Immunol.* **77**, 45–56.

11. Knippels, L. M. J., Penninks, A. H., Spanhaak, S., and Houben, G. F. (1998) *Clin. Exp. Allergy* **28**, 368–375.

12. Knippels, L. M. J., Penninks, A. H., and Houben, G. F. (1998) *J. Allergy Clin. Immunol.* **101**, 815–820.

13. Bernhisel-Broadbent, J., Dintzis, M. H., Dintzis, R. Z., and Sampson, H. A. (1994) *J. Allergy Clin. Immunol.* **93**, 1047–1059.

14. Ovary, Z. (1964) *Immunol. Methods*, 259–283.

15. Knippels, L. M. J., Penninks, A. H., Smit, J. J., and Houben, G. F. (1999) *Toxicol. Appl. Pharmacol.* **156**, 161–169.