

Oral sensitization to food proteins: a Brown Norway rat model

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Summary

Background Although several *in vivo* antigenicity assays using parenteral immunization are operational, no adequate enteral sensitization models are available to study food allergy and allergenicity of food proteins.

Objective This paper describes the development of an enteral model for food allergy research in the Brown Norway (BN) rat.

Methods The animals were exposed to ovalbumin either *ad libitum* via the drinking water (0.002 to 20 mg/mL) continuously for 6 weeks or by gavage (1 mg/mL per rat). Gavage dosing was performed either daily, twice a week, once a week or once every 2 weeks during a period of 6 weeks. No adjuvants were used during the sensitization studies.

Results After intra-gastric administration of ovalbumin once or twice a week or once every two weeks, no or only a very low frequency of ovalbumin-specific antibody responses were detected. Daily intra-gastric dosing with ovalbumin resulted in antigen-specific IgG as well as IgE responses in almost all animals tested. Upon *ad libitum* exposure, ovalbumin-specific IgG but no ovalbumin-specific IgE was detected. The cellular response was examined by determination of delayed-type hypersensitivity (DTH) reactions in the animals dosed by daily gavage and in the *ad libitum* exposed rats. Both sensitization protocols sensitized for DTH. The response was most pronounced in *ad libitum* exposed rats at day 28 of exposure.

Conclusions These studies show that the BN rat may provide a suitable animal model for inducing specific IgG and IgE responses as well as specific T-cell mediated hypersensitivity (DTH) to ovalbumin upon exposure via the enteral route without the use of adjuvants.

Keywords: food allergy, ovalbumin, oral, Brown Norway, rat, IgG, IgE, DTH

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Introduction

Humans rather frequently suffer from more or less severe allergic reactions after consumption of dietary proteins [1,2]. Type I or Immunoglobulin E (IgE)-mediated allergic reactions are known to play a major and primary role in food allergy [3]. No methods to predict whether a protein has a strong or weak potency of inducing food allergic reactions in susceptible humans are available at present.

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 [4–8]. Although effects upon 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. In mice, immune priming or sensitization may occur after enteral protein administration if adjuvants are used [9,10] or if enteral exposure is performed at early stages of life [9,11–13]. However, under normal conditions oral protein feeding of mice most easily results in tolerance induction [12,14–18]. This preferential response in mice indicates that the mouse is not a most suitable species for studying oral sensitization. For the guinea-pig, several studies with oral sensitization to food proteins have been described [19–24]. However, a significant difference in immunophysiology in guinea-pigs

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when compared with other species and the limited knowledge on the guinea-pig immune system and tools for studying the immun system are major draw backs for the use of this species in food allergy. For the rat, oral sensitization to food proteins administered through the diet or by intra-gastric dosing was also reported [25–27]. Although, sensitization to orally administered food proteins can be induced in rats, tolerance induction may also occur [28]. Nevertheless, 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. The Brown Norway (BN) rat is a high-immunoglobulin (particularly IgE) responder strain [29] and thus, to a certain degree, resembles atopic humans in their (genetic) predisposition to react with an overproduction of IgE to antigens. This preferential response indicates that the BN rat may be a suitable strain for food allergy research. Recently, Atkinson *et al.* [27] reported a Brown Norway (BN) rat model of food allergy using oral administration of ovalbumin in combination with intraperitoneal administration of carrageenan as an adjuvant to promote IgE production. We have reasoned that hyperstimulation of the IgE response in this way may influence the control mechanisms that occur under conditions of 'physiological' antigen exposure. Although such predispositional conditions may likely play a role in the development of food allergy in humans, we preferred the development of an enteral animal model without the use of an adjuvant. We hereby report an intra-gastric feeding protocol, without the use of an adjuvant, for inducing specific humoral (IgG and IgE) and cellular immune responses in the BN rat using the well defined chicken egg white allergen ovalbumin as a model antigen.

Materials and methods

Animals and maintenance

Young male Brown Norway (BN) rats 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 and had free access to food and tap-water. The rats were bred and raised on a commercially available ovalbumin-free rodent diet (SDS Special Diet Service type

RM3(E) FG SQC, Witham, UK). Pre-study blood samples were always tested for ovalbumin specific antibodies. All animal studies were approved by an independent ethical committee.

Experimental design

Animals, 4–6 weeks old at study initiation, were orally exposed to ovalbumin (OVA, Fluka Chemie, Buchs, Switzerland, purity: 70%). Several oral dosing protocols were applied. The animals were exposed to OVA either *ad libitum* via the drinking water (0.002, 0.02, 0.2, 2 or 20 mg/mL) continuously during 6 weeks or by gavage, using a 18-gauge stainless steel animal feeding needle (1 mg OVA/ml tap-water, 1 mL/animal). Gavage dosing was performed daily, twice a week, once a week, or once every 2 weeks during 6 weeks. The OVA containing drinking water was refreshed twice a day to avoid turning sour. After the induction period, the animals were not exposed to OVA for 1 week before being killed, except for the animals used to perform delayed type hypersensitivity tests. Control animals received normal drinking water and either or not a daily gastric intubation with 1 mL of tap-water. Blood samples were obtained from the orbital plexus under light CO₂ anaesthesia 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 2000 *g* and 4°C to obtain sera. The sera were stored at -20°C until analyses for anti-OVA specific IgG titres by Enzyme Linked Immunosorbent Assay (ELISA) and anti-OVA specific IgE by ELISA and passive cutaneous anaphylaxis (PCA)-test.

Positive control animals were injected intraperitoneally (i.p.) with 0.5 mL of a 0.2 mg/mL OVA solution in sterile saline on days 0, 2, 4, 7, 9 and 11. To potentiate the immune response, 0.2 mL of a 25 mg/mL AL(OH)₃ adjuvant suspension in sterile saline mixed with the 0.5 mL of OVA was injected on day 0. The animals were bled on day 28 by exsanguination from the abdominal aorta. Sera were prepared and stored as described before. Sera from positive control animals were pooled and used as positive control samples in the OVA-specific IgG and IgE ELISAs and in the PCA-test.

Assays for anti-OVA antibodies

Initially, only sera obtained from blood samples collected at day 28 and 42 were analysed for OVA-specific antibodies. At second instance (see results section), sera obtained from the *ad libitum* exposed animals were analysed for all time points (weeks 1–7). Serum IgG and IgE-specific for OVA were determined by ELISA. For the

detection of OVA-specific IgG, 96-wells microtitre 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%) in carbonate buffer, pH 9.6. The plates were washed three times with tap-water containing 0.4% Tween 20 (Merck, Hohenbrunn, Germany). This was followed by the addition of 100 µL/well phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA; Sigma Chemicals Co., St Louis, USA) 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 µL/well peroxidase conjugated goat anti-rat IgG (H + L) (Zymed, San Francisco, USA, diluted 1:500) in PBS/BSA-Tween 20 was added. After incubation for 1 h at 37°C, the plates were washed again and an enzyme substrate solution of 3,3',5,5'-tetramethylbenzidine (TMB; Sigma Chemicals Co., St Louis, USA, 100 µL/well; 6 mg/mL DMSO) was added. The plates were developed at room temperature for 5–15 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, Biorad Laboratories, Richmond, USA). A presera pool was used as negative control. The pooled presera 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 titre 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 titre. All analyses were performed in duplicate. The serum pool derived from the i.p. immunized animals was used as a positive control sample. Positive and negative control samples were incorporated for each 96-wells plate.

For the detection of OVA-specific IgE, 96-wells microtitre plates were coated overnight at 4°C with 100 µL/well mouse anti-rat IgE (MARE-1, Zymed, San Francisco, USA) 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 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 µL/well of an 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 h 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 h at 37°C, the plates were washed again and an enzyme substrate solution of TMB was added. Plate development, measurement and titre elaboration were as described for the OVA-specific IgG ELISA.

Passive cutaneous anaphylaxis

Passive cutaneous anaphylaxis (PCA) was tested essentially as described previously by Ovary *et al.* [30]. Naive (untreated) BN rats were shaven on the back and flanks and injected intradermally with 0.1 mL of the test sera in serial dilutions, followed 64 h later with an intravenous injection of 1 mL of a 1:1 mixture of a solution of OVA (Fluka Chemie, Buchs, Switzerland, purity: 70%, 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 titre 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 sera on each animal used for the PCA tests.

Measurement of delayed type hypersensitivity

Rats exposed to OVA *ad libitum* via the drinking water (20 mg/mL) or by daily gavage were tested for DTH reactions. At day 28 or 42 (separate groups of rats) of dosing, the DTH response was assessed by an ear swelling test. Rats received a subcutaneous injection of 25 µL OVA (Serva, Feinbiochemica, Heidelberg/New York, purity: >98%, 0–0.25 mg/mL) in PBS in one ear and 25 µL PBS in the contralateral ear. Increasements in ear thickness were measured 24 h after challenge using an electronic micrometer ($d = 0.01$ mm). OVA-specific DTH reactions were calculated by subtracting the ear thickness of the PBS-challenged ear from that of the OVA-challenged ear. DTH reactions were compared between naive (control) and sensitized animals. DTH responses of naive and sensitized animals were analysed for statistical significance of differences by two-tailed Student's *t*-test. Data were considered significantly different if $P < 0.05$.

Results

Negative controls

Pre-study blood samples were always tested for ovalbumin

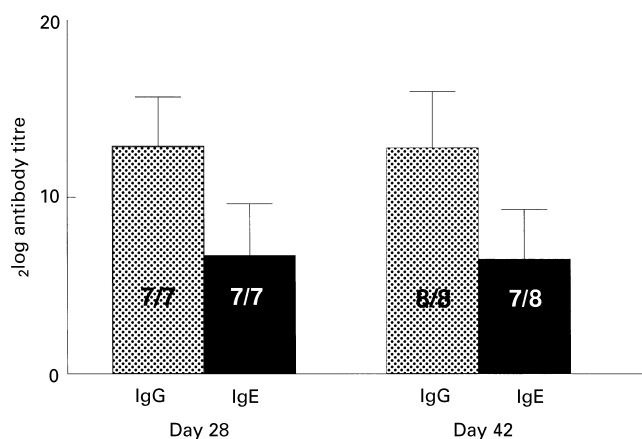


Fig. 1. Ovalbumin (OVA)-specific IgG and IgE titres upon daily intra-gastric dosing of young BN rats with OVA (1 mg/rat) for 28 or 42 days. The data are presented as mean $^2\log$ Ig titre $\pm$ SD of 7 (day 28) or 8 (day 42) rats per group. The number of animals developing an IgG or IgE response at the respective time-points are indicated in the bars.

(OVA) specific antibodies. No anti-OVA antibodies of the IgG or IgE class were detected in the pre-sera of the animals. These sera were pooled and used as negative control in the ELISA's and PCA tests.

Positive controls

OVA-specific IgG and IgE were demonstrated in the pooled day 28 serum from the positive control animals ($n = 8$, $^2\log$ IgG titre of 21 and $^2\log$ IgE titre of 15 in the OVA-specific ELISAs; data not shown). This serum pool was subsequently used as positive control in the ELISAs and PCA tests.

Administration of OVA by gavage

Rats exposed to OVA by gavage once a week ($n = 4$) or once every 2 weeks ($n = 4$) did not develop OVA-specific antibody responses. Upon intra-gastric dosing with OVA twice a week, one out of four animals developed an OVA-specific IgG ($^2\log$ titre 12 both at day 28 and 42) and an OVA-specific IgE response ($^2\log$ titre 3 at day 28 and $^2\log$ titre 8 at day 42) (data not shown). The administration of OVA by daily gavage induced an OVA-specific IgG and IgE response in almost all animals (Fig. 1). After 28 days of exposure, both OVA-specific IgG ($^2\log$ titre 12.9 ± 2.8 (mean $\pm$ SD), range $^2\log$ titre: 7–15) and OVA-specific IgE ($^2\log$ titre 6.7 ± 2.9 , range 2–9) were detectable in all seven tested animals. The same animals were used for measurement of delayed type hypersensitivity (DTH) reactions at day 28 (see section Delayed Type Hypersensitivity below). In a different group of animals exposed to OVA for

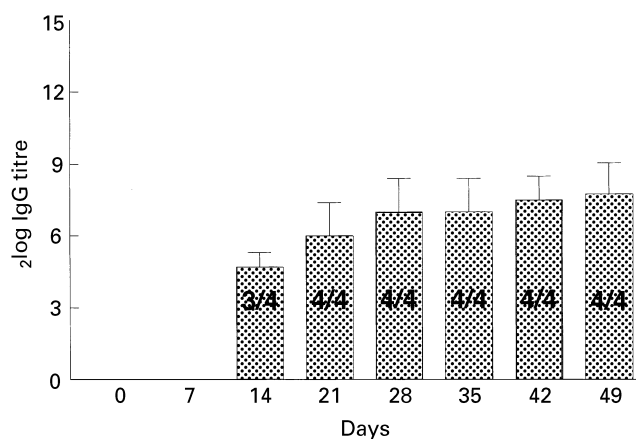


Fig. 2. Time dependency of ovalbumin (OVA)-specific IgG response in young BN rats upon *ad libitum* exposure to OVA (20 mg/mL) via drinking water for 42 days. After the induction period, the animals were not exposed to OVA for one week. IgG titres were determined in blood samples obtained at weekly intervals. The data are presented as mean $^2\log$ IgG titre $\pm$ SD of four rats per group. The number of animals developing an IgG response at the respective time-points are indicated in the bars. OVA-specific IgE could not be detected in animals exposed via drinking water.

42 days, OVA-specific IgG was detectable in all tested animals ($n = 8$, $^2\log$ titre 12.8 ± 3.2 , range 7–16) whereas OVA-specific IgE was detectable in seven out of eight animals ($^2\log$ titre in responders 6.5 ± 2.8 , range 2–9). The same animals were used for measurement of DTH reactions at day 42 (see below).

Ad libitum exposure to OVA via the drinking water

Antibody determinations by ELISA demonstrated that animals ($n = 4$) exposed to 0.002, 0.02 or 0.2 mg/mL OVA via the drinking water did not develop OVA-specific antibodies whereas exposure to 2 or 20 mg/mL OVA via the drinking water resulted in OVA-specific IgG responses ($^2\log$ titre 7 ± 1.4 , range 6–9 for 2 mg OVA/mL and $^2\log$ titre 7.8 ± 1.3 , range 6–9 for 20 mg OVA/mL, all data from sera collected at sacrifice). OVA-specific IgE could not be demonstrated in the day 28 and 42 samples from *ad libitum* exposed animals. Therefore, all serum samples obtained from the 20 mg/mL exposed animals (at weekly intervals) were analysed for OVA-specific antibodies. No OVA-specific IgE was detectable at any time-point investigated. OVA-specific IgG (Fig. 2) was first detectable at day 14 in 3 out of 4 animals ($^2\log$ titre 4.7 ± 0.6 , range 4–5). At day 21, OVA-specific IgG was detectable in all animals ($^2\log$ titre 6 ± 1.4 , range 4–7). Maximum titres were detected at day 28 ($^2\log$ titre 7 ± 1.4 , range 5 to 8). No significant changes in OVA-specific IgG titres occurred from day 28

Table 1. Comparison between ELISA and PCA results of representative sera (²log IgG and IgE/reaginic antibody titres in sera from ovalbumin sensitized rats)

Serum sample	IgG*	IgE*	PCA [†]
A	12	–	–
B	17	9	2
C	–	–	–
D	14	11	3
E	–	–	–
F	–	–	–
G	17	11	4
H	14	5	1
I	17	12	–
J	17	13	6
K	15	12	4

* Results of triplicate analysis; [†] results of duplicate PCA tests.

until termination of the induction period (day 42). In addition, OVA-specific IgG titres at day 49, one week after termination of the induction period, did not differ significantly from those seen at day 42. The average ²log titer was 7.8 ± 1.3 , with a range of 6–9 at termination of the study.

Passive cutaneous anaphylaxis test (PCA)

In order to confirm the OVA-specific IgE ELISA-results, several representative sera were also tested in a PCA test (Table 1). Sera that were positive in the OVA-specific IgE ELISA in general also showed positive PCA results, although the ELISA tended to be more sensitive. In some cases, sera that were positive in the IgE ELISA did not induce PCA responses. Competitive reactions due to high IgG titres may have played a role in this phenomenon. Sera negative in the ELISA all were also negative in the PCA test.

Delayed type hypersensitivity (DTH)

Possible priming for DTH responses in animals exposed to OVA via drinking water or by daily gavage was investigated by performance of ear swelling tests at day 28 and 42. Upon *ad libitum* exposure, strong DTH responses were measured at day 28 (Fig. 3a). In animals exposed to OVA via the drinking water for 42 days, significant DTH responses were also determined, but the responses were weaker than those observed at day 28. In intra-gastrically dosed animals no significant DTH responses were measured at day 28 (Fig. 3b). After 42 days of intra-gastric dosing, a DTH respon-

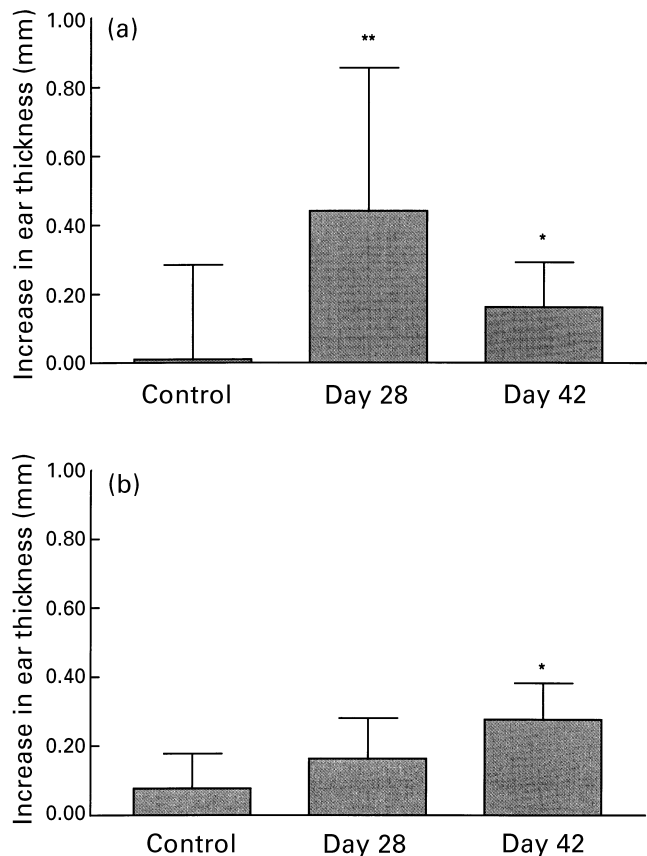


Fig. 3. DTH responses in animals *ad libitum* exposed to OVA via the drinking water (20 mg/mL) continuously for 28 or 42 days (a) or animals exposed to OVA (1 mg/rat) by daily gavage for 28 or 42 days (b). Control animals received tap-water *ad libitum* with or without a daily gavage with tap-water (1 mL/rat) for 28 or 42 days. The DTH responsiveness was determined by ear swelling test, OVA (6.25 µg in 25 µL PBS) was injected in one ear and 25 µL PBS in the contralateral ear. The data are presented as mean (differences in thickness between the OVA injected and contralateral ear) $\pm$ SD of eight rats per group. Statistics: two-tailed Student's *t*-test comparison between sensitized and control animals. **P* < 0.05, ***P* < 0.01.

siveness had developed which was comparable to the day 42 DTH responsiveness in animals exposed to OVA through the drinking water.

Discussion

In the present paper, studies on various oral dosing protocols to sensitize Brown Norway (BN) rats to ovalbumin (OVA) without the use of an adjuvant are presented. The most significant results are summarized in Table 2. From these results it appears that remarkable differences in immune responses to food antigens may occur depending on the dosing protocol applied. Administration of OVA *ad libitum*

Table 2. Overview on the examined parameters for humoral- and cellular immune responses at day 28 and 42 in BN rats exposed to ovalbumin either *ad libitum* via the drinking water or by daily gavage

	Ad libitum Day 28	Gavage Day 28	Ad libitum Day 42	Gavage Day 42
IgG response	++	++	++	++
IgE response	-	++	-	++
PCA	-	++	-	++
DTH	++	-	+	+

++, strongly positive; +, positive; -, negative.

via the drinking water (2 or 20 mg/mL) resulted in OVA-specific IgG production but no specific IgE, whereas daily intra-gastric dosing of 1 mg OVA resulted in OVA-specific IgG as well as OVA-specific IgE responses. Upon intra-gastric dosing with 1 mg OVA once every 2 weeks or once or twice a week, no or only a very low frequency of antibody induction was noted. Cellular immune responses were studied by performing DTH tests. The DTH responses were most pronounced in *ad libitum* exposed animals at day 28. At day 42, both sensitization regimes primed for comparable DTH responses, but the responses were weaker than the day 28 DTH responsiveness in *ad libitum* exposed rats.

The strong DTH responsiveness in *ad libitum* exposed rats associated with the absence of an IgE response suggests a dichotomy in T-cell function as is also observed in mice and humans. In the latter species, CD4⁺ T helper (TH₁) cells, producing interleukin-2 (IL-2) and interferon-gamma (IFN γ), promote cellular immune responses and, to a certain extent, IgG production [31–33]. Activated CD4⁺ T helper-2 (TH₂) cells, producing IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, strongly support humoral immune responses, particularly IgE responses [32,33]. Similarly, for the rat there is also evidence, although not as pronounced as for mice, for a functional dichotomy in TH cell functions [34,35], with IL-4 and IFN γ having the same crossregulatory function as in the mouse [36]. In the *ad libitum* exposed animals in the present studies, specific IgG but no specific IgE was detected. This suggests a TH₁-like response, which is in accordance with the pronounced DTH response seen in these animals. In the gavage dosed animals, a TH₂-like response seems to dominate with pronounced specific IgE and IgG responses and a weaker DTH responsiveness. However, this suggested dichotomy is not reflected in differences in DTH responsiveness upon prolonged exposure, since there was no difference in DTH reaction between *ad libitum* and gavage dosed animals at day 42.

Various factors may play a role in the development of the distinct immune responses upon *ad libitum* and gavage administration of food proteins. Estimation of the OVA intake by the *ad libitum* exposed rats indicates and OVA intake of 0.2 and 2 g/kg bodyweight (BW)/day for 2 and 20 mg/mL exposed rats respectively, vs approximately 5 mg/kg BW/day for the gavage dosed rats, based on a default liquid consumption figure of 100 g/kg BW/day. However, *ad libitum* exposure to 0.002, 0.02 and 0.2 mg/mL OVA corresponding to an estimated OVA intake of 0.2, 2 and 20 mg/kg BW/day respectively, (assuming a liquid intake of 100 mL/kg BW), comparable to the 5 mg/kg BW/day for gavage dosing, did not induce antibody production. Therefore, a difference in OVA intake is not likely to account for the observed differences in immune responses. A factor that may have played a role in the differences in immune responses upon gavage and *ad libitum* exposure to OVA is a difference in the OVA intake over the day. With gavage dosing, the animals received a daily bulk dose of OVA whereas upon *ad libitum* exposure, a more gradual intake of OVA over a prolonged period will have occurred. This difference may (in part) account for the observed differences between the intra-gastrically and *ad libitum* exposed animals. However, several other factors may have played a role in the revealed differences [25,26,37]. One of the factors which influences the potential immunological response to ingested protein is gastro-intestinal digestion as was shown by Hanson *et al.* [38]. Prefeeding of an endopeptidase inhibitor to mice reversed the outcome of intra-gastric protein feeding to mice from the induction of systemic non-responsiveness to priming for later protein-specific antibody responses. Moreover, studies by Catto-Smith *et al.* [39] demonstrated that intra-gastric administration of proteins may interfere with normal gastric functions such as the gastric emptying rate and as such may affect the digestive breakdown. An altered digestive breakdown of food proteins may result in a different spectrum of digestive fragments. Such differences may also (in part) account for the observed differences upon intra-gastric dosing and *ad libitum* exposure to OVA. At present no data are available to definitively explain the observed differences between the several intra-gastric dosing protocols. However, a difference in antigen load may well account for the differences seen since only daily intra-gastric dosing resulted in specific IgG and IgE responses whereas upon less frequent intra-gastric administration (i.e. lower antigen load) no or only a very low frequency of antibody induction was noted.

Our studies show that the BN rat can be sensitized by the enteral route without the use of an adjuvant by daily gavage dosing with an antigen. Less frequent administration of 1 mg of OVA by gavage (once every two weeks or once or twice a week) in general did not induce specific IgG or specific IgE antibody responses. Recently, a study was

reported [27] in which administration of 10 mg OVA by gavage twice a week in combination with intraperitoneal administration of carrageenan as an adjuvant induced specific IgE resulting in levels of reagenic antibodies comparable to the levels induced upon daily intra-gastric dosing of 1 mg OVA, without the use of adjuvant, in our studies. It remains to be elucidated whether the observed differences are due to the applied dose levels of OVA or to the use of the adjuvant. In the studies reported by Atkinson *et al.* [27] intra-gastric administration of 10 mg OVA twice a week resulted in responder percentages of about 60–100%. We performed several successive studies in which we sensitized BN rats by daily gavage with 1 mg OVA. The percentage of IgE responders in our studies in general exceeded 80%. However, occasionally, no OVA-specific IgE responses were induced upon daily gavage dosing with OVA in our studies. One of the major factors that may negatively affect the results of oral sensitization studies is unscheduled dietary pre-exposure of the test animal or their parental generations to the antigen under investigation. For guinea-pigs [23] and rats [Knippels *et al.* in preparation], it was demonstrated that dietary exposure of animals to the proteins under investigation affects the results of oral sensitization studies with the offspring and that at least two generations of animals have to be bred on a specified antigen free diet to avoid any influences in this respect. As far as we could reveal, the animals used in our studies met this condition, since pre-study blood samples evaluated on a routine basis prior to the initiation of our studies were always negative for OVA-specific IgG and IgE antibodies. Although we have not been able to determine the cause why sensitization did not occur in some of our experiments, the same phenomenon was reported from oral sensitization studies with rats performed by Jarrett *et al.* [26].

The studies reported here show that the BN rat provides a suitable animal model for inducing specific IgG and IgE responses as well as specific T-cell mediated hypersensitivity (DTH) upon daily intra-gastric dosing of 1 mg OVA without the use of an adjuvant. Currently, studies are in progress to investigate differences in oral sensitizing potentials of various food proteins using the model reported in this paper and to qualitatively and (semi)quantitatively compare the results with human clinical data. In addition, studies are in progress to characterize the rat model in more detail with respect to immune-mediated effects upon challenge and to study mechanisms involved in sensitization.

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References

- 1 Kajoosari M. Food allergy in Finnish children aged 1 to 6 years. *Acta Paediatr Scand* 1982; 71:815–9.
- 2 Kardinaal AFM. Epidemiology of food allergy and food intolerance. In: Somogyi JC, Müller HR, Ockhuizen TH, eds. *Food allergy and food intolerance. Nutritional aspects and developments*. Basel: Karger, 1991: 105–15.
- 3 Bruijnzeel-Koomen C, Ortolani C, Aas K et al. Adverse reactions to food. *Allergy* 1995; 50:623–35.
- 4 Byars NE, Ferraresi RW. Intestinal anaphylaxis in the rat as a model of food allergy. *Clin Exp Immunol* 1976; 24:352–6.
- 5 Freier S, Eran M, Goldstein R. The effect of immediate type gastrointestinal allergic reactions on brush border enzymes and gut morphology in the rat. *Pediatr Research* 1985; 19:456–59.
- 6 Müller K, Nicklin S. Mechanisms of food intolerances: development and use of experimental animal models. In: Walker R, Quattrucci E, eds. *Nutritional and Toxicological aspects of food processing*. 1988: 351–64.
- 7 Turner MW, Barnett GE, Strobel S. Mucosal mast cell activation patterns in the rat following repeated feeding of antigen. *Clin Exp Allergy* 1990; 20:421–7.
- 8 Curtis GH, Patrick MK, Catto-Smith AG, Gall DG. Intestinal anaphylaxis in the rat. Effect of chronic antigen exposure. *Gastroenterology* 1990; 98:1558–66.
- 9 Challacombe SJ, Tomasi TB. Systemic tolerance and secretory immunity after oral immunization. *J Exp Med* 1980; 152:1459–72.
- 10 Bozelka BE, Carrow E, Pahud JJ, Lehrer SB. Soy- and shrimp-specific IgE responses in orally and intraperitoneally immunized mice. *Int Arch Allergy Appl Immunol* 1987; 83:271–7.
- 11 Hanson DG. Ontogeny of orally induced tolerance to soluble proteins in mice I. Priming and tolerance in newborns. *J Immunol* 1981; 127:1518–24.
- 12 Strobel S, Ferguson A. Immune responses to fed protein antigens in mice. Systemic tolerance or priming is related to age at which antigen is first encountered. *Pediatr Research* 1984; 18:588–94.
- 13 Strobel S, Ferguson A. Modulation of intestinal and systemic immune responses to a fed protein antigen in mice. *Gut* 1986; 27:829–37.
- 14 Ngan J, Kind LS. Suppressor T cells for IgE in Peyer's patches of mice made tolerant by the oral administration of ovalbumin. *J Immunol* 1978; 120:861–5.
- 15 Mowat AM. The regulation of the immune responses to dietary protein antigens. *Immunol Today* 1987; 8:93–8.
- 16 Stokes CR, Newby TL, Bourne FJ. The influence of oral immunization on local and systemic immune responses to heterologous antigens. *Clin Exp Immunol* 1983; 52:399–406.
- 17 Melamed D, Friedman A. In vivo tolerization of Th1 lymphocytes following a single feeding with ovalbumin: anergy in the absence of suppression. *Eur J Immunol* 1994; 24:1974–81.

- 18 Friedman A, Al-Sabbagh A, Santos LMB et al. Oral tolerance: a biologically relevant pathway to generate peripheral tolerance against external and self antigens. *Chem Immunol* 1994; 58:259–90.
- 19 Devey ME, Anderson KJ, Coombs RRA. The modified anaphylaxis hypothesis for cot death. Anaphylactic sensitization in guinea-pigs fed cow's milk. *Clin Exp Immunol* 1976; 26:542–8.
- 20 Coombs RRA, Bevey ME, Andersom KJ. Refractoriness to anaphylactic shock after continuous feeding of cows' milk to guinea pigs. *Clin Exp Immunol* 1978; 32:263–71.
- 21 Andersson KJ, McLaughlan P, Devey ME, Coombs RRA. Anaphylactic sensitivity of guinea-pigs drinking different preparations of cows' milk and infant formulae. *Clin Exp Immunol* 1979; 35:454–61.
- 22 McLaughlan P, Anderson KJ, Widdowson EM, Coombs RRA. Effect of heat on the anaphylactic-sensitising capacity of cows' milk, goats' milk, and various infant formulae fed to guinea-pigs. *Arch Dis Child* 1981; 56:165–71.
- 23 Pahud JJ, Schwarz K. Oral sensitization to food proteins in animal models, a basis for the development of hypoallergenic infant formula. In: Production, regulation, and analysis of infant formula. Proc. of the Tropical Conference, Virginia, 1985, Association of Official Analytical Chemists, USA, 1985, 264–71.
- 24 Piacentini GL, Bertolini A, Spezia E, Piscione T, Boner AL. Ability of a new infant formula prepared from partially hydrolyzed whey to induce anaphylactic sensitization; evaluation in a guinea pig model. *Allergy* 1994; 49:361–64.
- 25 Bazin H, Platteau B. Production of circulating reaginic (IgE) antibodies by oral administration of ovalbumin to rats. *Immunology* 1976; 30:679–84.
- 26 Jarrett EEE, Haig DM, McDougall W, McNulty E. Rat IgE production. II. Primary and booster reaginic antibody responses following intradermal or oral immunization. *Immunology* 1976; 30:671–7.
- 27 Atkinson HAC, Johnson IT, Gee JM, Grigoriadou F, Miller K. Brown Norway rat model of food allergy: Effect of plant components on the development of oral sensitization. *Fd Chem Toxic* 1996; 34:27–32.
- 28 Steinmann J, Wottge HU, Muller-Ruchholtz. Immunogenicity testing of food proteins: *in vitro* and *in vivo* trials in rats. *Int Arch Allergy Appl Immunol* 1990; 91:62–5.
- 29 Holt P, Turner KJ. Persistence of IgE secreting cells that are refractory to T-cell control. *Int. Arch Allergy Appl Immunol* 1985; 77:45–56.
- 30 Ovary Z. Passive cutaneous anaphylaxis. *Immunol Methods* 1964; 259–83.
- 31 Mosmann TR, Cherwinski HM, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 1986; 136:2348–57.
- 32 Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989; 7:145–73.
- 33 Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 1996; 17:138–46.
- 34 McKnight AJ, Barclay AN, Mason DW. Molecular cloning of rat interleukin 4 cDNA analysis of the cytokine repertoire of subsets of CD4+ T cells. *Eur J Immunol* 1991; 21:1187–94.
- 35 Papp I, Wieder KJ, Sablinski T et al. Evidence for functional heterogeneity of rat CD4+ T cells *in vivo*. Differential expression of IL-2 and IL-4 mRNA in recipients of cardiac allografts. *J Immunol* 1992; 148:1308–14.
- 36 Noble A, Staynov DZ, Kemeny DM. Generation of rat Th2-like cells *in vitro* is interleukin-4-dependent and inhibited by interferon- γ . *Immunology* 1993; 79:562–7.
- 37 Pauwels R, Bazin H, Platteau B, Van der Straeten M. The effect of age on IgE production in rats. *Immunology* 1979; 36:145–59.
- 38 Hanson DG, Roy MJ, Green GM, Miller SD. Inhibition of oral induced immune tolerance in mice by prefeeding an endopeptidase inhibitor. *Reg Immunol* 1993; 5:76–84.
- 39 Catto-Smith AG, Patrick MK, Scott RB, Davidson JS, Gall DG. Gastric response to mucosal IgE-mediated reactions. *Am J Physiol* 1989; 257:G704–8.