



Brown Norway Rat Model of Food Allergy: Effect of Plant Components on the Development of Oral Sensitization

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Abstract—The Brown Norway (BN) rat was examined as a model for investigating factors that influence the development of food allergy. An antigen dose-response curve for the production of antigen-specific reagenic antibody (IgE) induced through the oral route was determined. Animals were dosed orally with 1.0, 2.5, 5.0, 7.5, 10.0 and 12.0 mg ovalbumin/ml (0.5 ml/100 g twice a week for 6 wk). To promote IgE production the adjuvant carrageenan was administered once a week by the ip route. The effect on oral sensitization of 1.5 mg *Gypsophila* sp. saponin/ml administered together with the antigen on oral sensitization was examined in animals treated with 2.5, 6.0 or 10.0 mg ovalbumin/ml. The number of animals producing antigen specific reagenic antibody in response to 2.5 mg ovalbumin/ml was significantly increased ($P < 0.01$) in the group that received saponin with 2.5 mg ovalbumin/ml. These studies indicate that the BN rat is a sensitive model for the investigation of allergic reactions to food and has the potential to determine the impact of other dietary factors on the development of oral sensitization.

INTRODUCTION

The development of immunoglobulin E (IgE)-mediated allergic reactions to foods occurs as a result of the breakdown of normal immune tolerance. There has been increasing interest and concern about the 'allergic' reactions to certain types of food, and the influence of other dietary components or factors on the development of allergy and allergic breakthrough. In general terms, if healthy immunocompetent animals are exposed to protein antigens (prototype allergens) through natural exposure (i.e. by feeding or inhalation), their normal baseline immune response is the induction of immunological tolerance (Mowat, 1987). The effect of dietary components on intestinal integrity may be a factor in the modulation of gastrointestinal immune responses. Substances in food, such as glycoalkaloids and saponins, can increase gastrointestinal permeability (Ridout *et al.*, 1988). Increased uptake of allergen due to materials in the diet that affect intestinal permeability may increase the likelihood of the development of food allergy. The type and concentration of antigen reaching the immune system has been shown to be an important factor in determining the development of

immune responses to mucosal antigens in animal models (Strobel, 1986).

Information gained from animal experimental models has revealed the extent to which gut immune responses depend on age, dose and frequency of antigen administration, as well as the conditions needed to induce vigorous mucosal immune reactions (Challacombe *et al.*, 1987; Dahlgren *et al.*, 1991). In general, research directed towards understanding the development of mucosal immune responses to ingested proteins has addressed the induction and suppression of IgG rather than IgE (reagenic) antibody responses or the immunogenicity rather than the allergenicity (Enomoto *et al.*, 1993; Steinmann *et al.*, 1990). Although experimental models of IgE-mediated hypersensitivity reactions have demonstrated the effects of allergic reactions on mucosal morphology and function (Strobel, 1991; Strobel and Ferguson, 1986), there are as yet no established animal models available for screening potentially allergenic food proteins through the natural route of feeding. Such a model would have applications for the assessment of the allergenicity of new products or those specifically modified to reduce allergenicity, as well as the influence of other elements of the diet on the development of sensitization.

The development of allergies is associated with the genetic predisposition to develop IgE antibody responses (atopy). Although there are no naturally occurring animal models of the atopic state, there

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Abbreviations: ADE = area of dye extravasation; BN = Brown Norway; CGN = carrageenan; IEV = intestinal extravasation; PBS = phosphate buffered saline; PCA = passive cutaneous anaphylaxis.

exist high IgE responder strains of rat such as the Brown Norway (BN). The BN rat has been used in several laboratories for investigating aspects of allergic responses (Atkinson and Miller, 1994; Eidleman *et al.*, 1988; McMenamin and Holt, 1993; Miller and Nicklin, 1988). The development of a rat model would have the added advantage that it could easily be integrated into toxicological safety evaluation programmes, which make extensive use of rat (rodent) models.

Adjuvants are usually included in experimental models of allergy as these increase the capacity of the animal to produce antigen-specific responses. Carrageenan (CGN) has been found to be a suitable adjuvant for the induction of reagenic antibody since it primes for both primary and secondary responses (Nicklin *et al.*, 1985). The magnitude of the reagenic response is influenced not only by the dose of antigen used but also by the timing of CGN administration relative to the antigen (Nicklin *et al.*, 1985 and 1988). Using this adjuvant and the BN rat, the profile of antibody specificity to the complex mixture of allergens present in milk was found to be similar to that in humans (Atkinson and Miller 1994).

In order to elucidate the relationship between substances capable of increasing the permeability of the gastrointestinal mucosa and the development of allergic reactions to food antigens, it was first necessary to develop a treatment regimen for inducing sensitization by the oral route. Ovalbumin was used as a standard allergen (as it is one of the most common food allergens in humans). The plant-derived bioactive compound saponin from *Gypsophila* sp. was used to determine whether saponin renders the epithelium more permeable to the allergen, and as a consequence is capable of promoting the induction of sensitization. *In vitro* studies of the effects of *Gypsophila* sp. saponin on mammalian small intestine permeability have indicated that it increases the permeability of small intestine mucosal cells, while inhibiting active transport, and facilitates uptake of materials to which the gut would not normally be permeable (Johnson *et al.*, 1986). We now report a regimen for inducing reagenic antibody responses to orally administered ovalbumin in the BN rat strain and the modulation of this response by saponin.

MATERIALS AND METHODS

Animals

Brown Norway (BN) rats (male, 6–8 wk old), obtained from Bantin and Kingman (Hull, Humberside, UK), were used for immunization procedures, while Sprague–Dawley rats (male, 6–8 wk old), obtained from Harlan Olac, 1976 (Bicester, UK), were used for analysis of reagenic antibody. Animals received a nutritionally adequate diet, either Rat and Mouse No. 1 expanded, supplied by Special Diet Services Ltd (Whitham, Essex, UK) (SDS), or a milk- and ovalbumin-free diet BeeKay (BK) supplied

by Bantin and Kingman. Both food and tap water were freely available.

Test materials

Ovalbumin (Sigma Ltd, Poole, Dorset, UK) was used for sensitization. Carrageenan Type V (CGN; Sigma) was used as an adjuvant. *Gypsophila* sp. saponin (Sigma) was treated to remove non-saponin material using reverse phase chromatography (AFRC Institute of Food Research, Norwich, UK).

Reagenic antibody analysis

The presence of reagenic antibody was assessed by passive cutaneous anaphylaxis assay (PCA). Naive (untreated) Sprague–Dawley rats were shaved on the back and flanks and injected intradermally with 0.1 ml of the test sera. 24 hr later each rat was injected iv with 0.6 ml ovalbumin (1 mg/ml) together with 0.4 ml Evans blue (2% in saline). After 20–30 min the animal was examined for positive responses. The diameter of dye extravasation at the site of serum injection was measured and the area of dye extravasation (ADE) calculated.

IgG antibody analysis

Antigen-specific IgG was determined using an ELISA technique. Ovalbumin (10 µg/ml in 50 mM carbonate buffer pH 9.6) was bound to an ELISA plate (M129B Dynatech Laboratories Ltd, Billingshurst, Sussex, UK) for 3 hr at 37°C. Excess antigen or any other material was removed by washing five times after this and each subsequent incubation, using phosphate buffered saline (PBS) containing 0.5% Tween 20 and 2% sheep serum albumin (Sigma). Residual binding sites were blocked with goat serum (Seralab, Crawley Down, Sussex, UK) in carbonate buffer for 1 hr at 37°C. Sera to be examined were diluted 1/125 in PBS, then titrated using log₂ dilutions, and incubated overnight at 4°C. Non-specific binding sites were blocked with sheep serum (Serotec Ltd, Kidlington, Oxford, UK) prior to incubation with peroxidase-labelled sheep anti-rat IgG Fc antibody (Serotec) in 0.05% Tween 20 in PBS for 1 hr at room temperature. Substrate (*o*-phenylene diamine, 1.08 mg/ml, and 20 volumes H₂O₂, 1 µl/ml) was added to develop the colour. The reaction was stopped using 0.1 M HCl and the optical density read on a Luminar ELISA reader at 490 nm. The level of IgG was reported as a percentage of an anti-ovalbumin reference serum.

Intestinal extravasation assay

The effect of saponin on intestinal permeability was determined using the intestinal extravasation assay (Nicklin *et al.*, 1986). Briefly, groups of three non-sensitized BN rats were starved overnight prior to gavage with 2 ml saponin at concentrations from 0.01 to 5 mg/ml in distilled water. Control animals received distilled water alone. They were then injected iv with ¹²⁵I-labelled rat albumin (approximately

5×10^6 dpm in 100 μ l PBS/100 g body weight). After 40 min, animals were killed by cervical dislocation and a 50- μ l blood sample taken from the retro-orbital sinus immediately *post mortem*. The small intestine

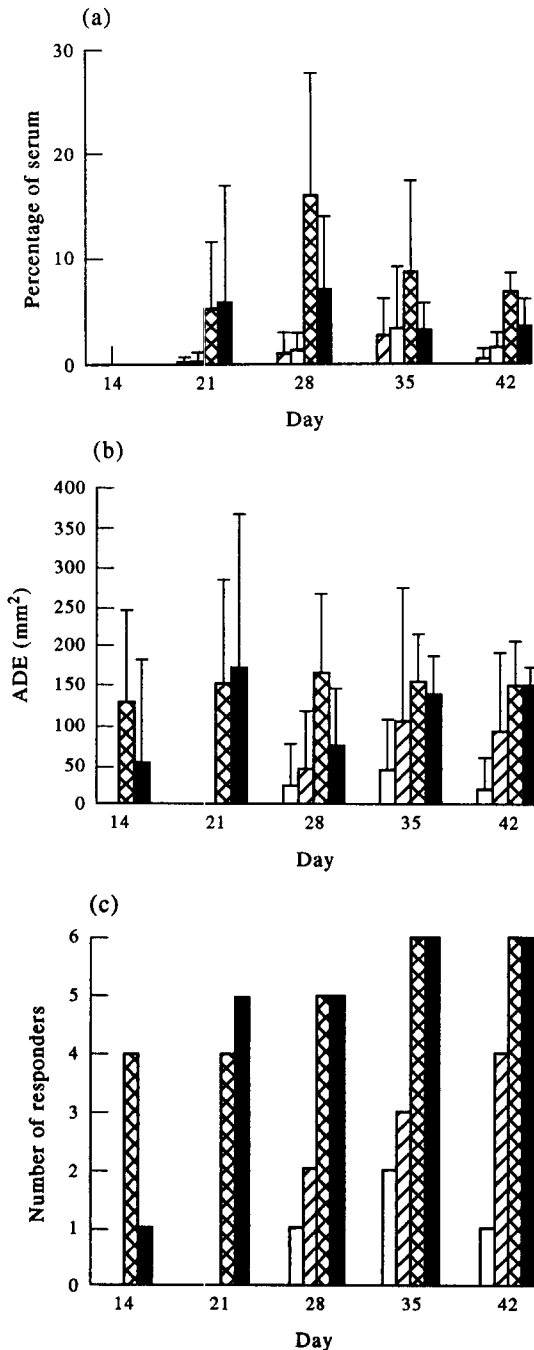


Fig. 1. Effect of dose of allergen on the development of antigen-specific IgG and reagenic antibody responses in groups of six BN rats receiving ovalbumin orally twice a week: 5.0 mg/ml (□), 7.5 mg/ml (▨), 10.0 mg/ml (▩) and 12.5 mg/ml (■). (a) IgG responses expressed as a percentage of an anti-ovalbumin reference serum; values are means $\pm$ SD. (b) Reagenic antibody responses were assessed by passive cutaneous anaphylaxis (PCA) expressed as mean $\pm$ SD of area of dye extravasation (ADE). (c) Number of animals developing reagenic responses, as determined by the PCA assay.

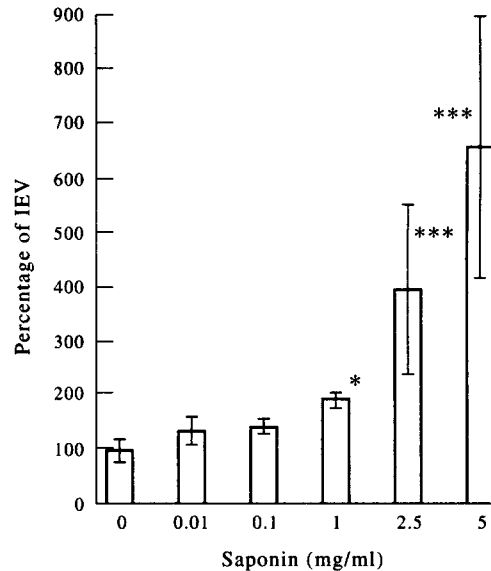


Fig. 2. Effect of various concentrations of *Gypsophila* sp. saponin on intestinal permeability. Results are expressed as a percentage of the control group intestinal extravasation index (IEV). Values are means $\pm$ SD for groups of three BN rats. Asterisks indicate significant differences compared with the control (* P < 0.05; *** P < 0.001; least significant difference test).

was removed and the intestinal contents removed by flushing with 2 ml warm saline. The radioactivity within the blood and intestinal washings was determined using a Packard 5230 auto- γ scintillation spectrophotometer (Packard Instrument Corp). Results are presented as the intestinal extravasation (IEV) index: IEV index = (dpm in intestinal washings)/(dpm in 50 μ l blood).

Treatment

Groups of six BN rats were dosed orally (0.5 ml/100 g body weight) with solutions of 1.0, 2.5, 5.0, 7.5, 10.0 or 12.5 mg ovalbumin/ml in distilled water twice weekly for 6 wk. In addition, they received an injection of CGN (1 mg/ml in saline) once a week for 6 wk. Animals were bled at weekly intervals from the lateral tail vein. Sera were assessed for the presence of anti-ovalbumin-specific reagenic antibody by PCA and anti-ovalbumin-specific reagenic antibody IgG by ELISA.

The effect of saponin on oral sensitization was assessed using a dose of saponin that produced a twofold increase of the background level of intestinal extravasation. Groups of eight animals were dosed orally twice weekly (0.5 ml/100 g body weight) with 2.5, 6.0 or 10 mg ovalbumin/ml in the presence or absence of 1.5 mg saponin/ml. They also received 1 mg CGN ip once a week. Animals were bled at weekly intervals and the sera assessed for the presence of ovalbumin-specific reagenic antibody.

Table 1. Effect of saponin on number of BN rats developing reagenic antibody responses following oral exposure to ovalbumin

Ovalbumin (mg/ml)	Saponin (1.5 mg/ml)	No. of responders on day				
		14	21	28	35	42
2.5	—	0/8	0/8	0/8	0/8	0/8
	+	2/8	5/8*	6/8**	6/8**	6/8**
6.0	—	0/8	2/8	2/8	2/8	2/8
	+	1/8	5/8	6/8	6/8	6/8
10.0	—	3/8	5/8	5/8	5/8	5/8††
	+	3/8	4/8	4/8	5/8	6/8

Asterisks indicate significant differences between saponin-treated and control groups (* $P < 0.05$; ** $P < 0.01$; Fisher's exact test). Daggers indicate significant difference between ovalbumin dose levels (†† $P < 0.01$; Fisher's exact test).

RESULTS

The administration of ovalbumin by the oral route induced both antigen-specific IgG and reagenic antibody (Fig. 1a,b). The levels of IgG antibody present could be detected from day 21 onward in dose levels of 5 mg/ml and above. The maximum response was seen at day 28. The reagenic response differed in that it could be detected from day 14 onward in animals given the 10.0 and 12.0 mg/ml dose levels, and in those receiving 5 mg/ml and above from day 28 onward. Figure 1c shows the number of animals developing reagenic responses in each group. The

lowest level of allergen that induced reagenic responses was 5 mg/ml. In both the 10 and 12.0 mg/ml dose levels six out of six responders had been achieved by day 42.

The effect of saponin on intestinal permeability is shown in Fig. 2. The level of movement of the ^{125}I -labelled albumin into the intestinal lumen was increased in the presence of saponin at the 2.5 and 5 mg/ml dose levels ($P < 0.001$) and at the 1 mg/ml dose level ($P < 0.05$). The effect of saponin on oral sensitization is shown in Table 1, and the individual antigen-specific reagenic antibody responses are shown in Fig. 3. The numbers of animals developing

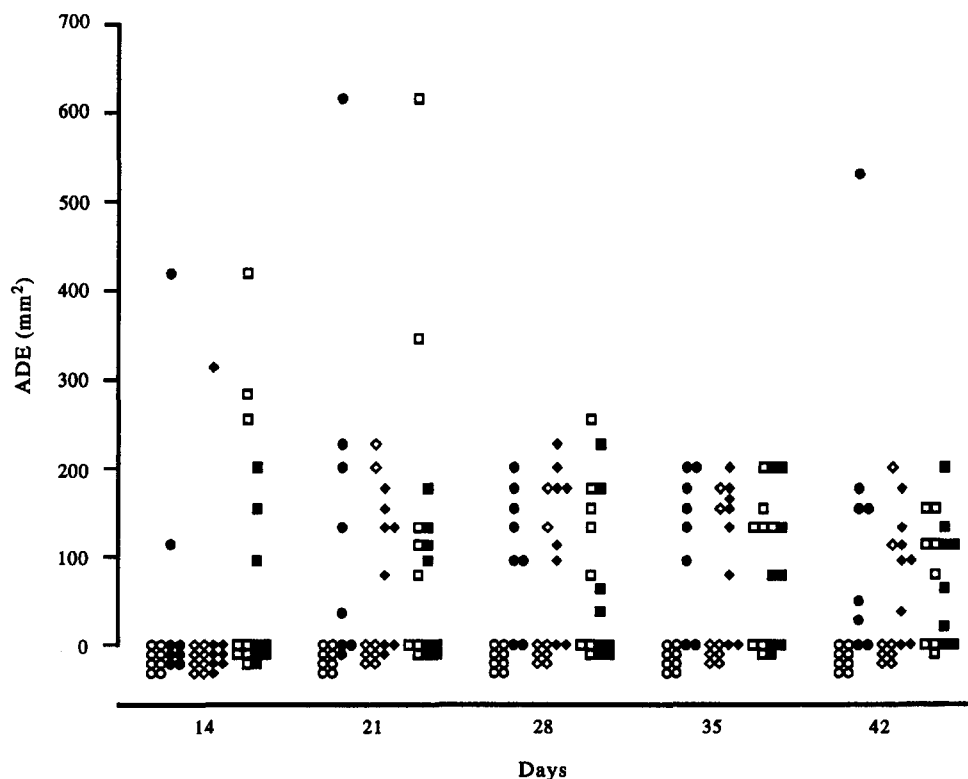


Fig. 3. Individual antigen-specific reagenic antibody responses from groups of eight BN rats receiving ovalbumin orally twice a week: 2.5 mg/ml (○), 6.0 mg/ml (◇) and 10.0 mg/ml (□) alone, and 2.5 mg/ml (●), 6.0 mg/ml (◆) and 10.0 mg/ml (■) in the presence of 1.5 mg saponin/ml. Responses were assessed by passive cutaneous anaphylaxis and expressed as the area of dye extravasation (ADE).

reaginic responses when receiving ovalbumin in the absence of saponin was increased from none of eight in animals given 2.5 mg/ml compared with five of eight in the group given 10 mg/ml ($P < 0.05$). In the presence of saponin this was increased to six of eight for all ovalbumin doses by day 42. In the lowest dose level of 2.5 mg/ml, reaginic antibody production was induced in five of eight animals by day 21 ($P < 0.05$) and six of eight from day 28 onward ($P < 0.01$). These results show that saponin administered together with ovalbumin could induce sensitization at levels that normally did not give rise to reaginic antibody production.

DISCUSSION

Exposure to antigen by the mucosal route is generally directed towards the development of tolerance rather than sensitization. As induction of IgE responses is under tight regulation, the choice of the appropriate rat strain and adjuvant is an important factor in the development of a suitable animal model. The way in which adjuvants work differ: some, such as alum, require physical interaction with the allergen, whereas it has been suggested that CGN affects IgE production through depressing the production of IFN- γ , a cytokine involved in down-regulation of IgE responses (Suzuki *et al.*, 1986). This is particularly important when considering sensitization by the oral route because interaction of adjuvant with allergen could alter allergen uptake across the intestinal mucosa.

The response of the BN rat to oral ovalbumin was found to be dose dependent, which influenced the numbers of animals developing reaginic responses. It was clear that repeated doses were required to induce oral sensitization, particularly at the lower dose levels. In the present studies the onset of reaginic antibody production occurred earlier at the high dose levels, and it is possible that, if the treatment regimen had been extended, six out of six responders might also have been produced in a group receiving 7.5 mg ovalbumin/ml. The presence of serum IgG responses is usually taken as an indicator of immune recognition and this was also absent at the lower dose levels. These results support the concept that the level of allergen in the diet is an important factor in the development of sensitization.

The identification of a dose response to orally administered allergen is an important finding because it allows the impact of other dietary factors on the development of oral sensitization to be examined. Using high, medium and low doses of ovalbumin it was possible to demonstrate the effect of a material that increases intestinal permeability on the development of oral sensitization. Thus, saponin was found to have a marked effect on the induction of oral sensitization at the dose level of 2.5 mg ovalbumin/ml, which did not normally induce sensitization. The number of treatments required to induce reaginic

antibody production was also reduced when saponin was administered together with ovalbumin. Although the acute effect of saponin in the extravasation assay was to increase movement of ^{125}I -labelled albumin into the gut lumen, other studies examining the effect of saponin on elicitation of allergic responses in sensitized BN rats have shown that oral exposure to this level of saponin in conjunction with allergen can increase the severity of local allergic reactions (Gee *et al.*, 1994). This observation, together with the current findings, suggest that increased levels of allergen are available to the immune system. The effect of saponin may also be related to the antigen being seen by the immune system after a different route of transport across the mucosa (Johnson *et al.*, 1986). As orally fed *Quillaja* saponin has been shown to amplify the immunopotentiating ability of ip-administered inactive rabies vaccine in mice, there is also the possibility that the related *Gypsophila* sp. saponin may act as an adjuvant in its own right (Chavali *et al.*, 1988).

The level of saponin that was administered in these studies was 7.5 mg/kg body weight/day on each day of treatment, which is similar to the estimated intake of 6.13 mg saponins/kg body weight/day from various plant foods by British adults (Ridout *et al.*, 1988). This suggests that exposure to membranolytic food constituents is undesirable even at a level that might be judged safe by the standards of conventional toxicology. However, the level of exposure that entails an increased risk of sensitization remains to be established in trials with human subjects.

These studies have shown that the BN rat model could provide a suitable experimental animal model for the investigation of dietary factors influencing the development of oral sensitization. The advantage of an animal model in which a dose response to allergen can be obtained means that we have the potential to study factors that protect as well as enhance sensitization. In addition to assessing the allergenicity of new products or those specifically modified to reduce allergenicity, the BN rat system could be used to establish the contribution of various dietary components to the development of food allergy.

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