

Characterization of antibody responses induced in rodents by exposure to food proteins: influence of route of exposure

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Abstract

There is a growing interest in the development of methods to characterize the allergenic properties of novel proteins, particularly those expressed by transgenic crop plants. Approaches to the direct evaluation of allergenic potential have focused generally on the ability of proteins to induce antibody (particularly IgE antibody) after systemic (intraperitoneal; i.p.) or gavage administration to high IgE responder strain rodents. To date there has been no systematic comparison of the reliability, sensitivity or selectivity of these approaches. We have, therefore, compared antibody (IgG and IgE) responses induced in Brown Norway (BN) rats by daily gavage administration and in BALB/c strain mice following intraperitoneal or gavage exposure to food proteins of varying allergenic potential. Animals were exposed to the allergens peanut agglutinin and ovalbumin (OVA) or to a crude potato protein extract (PPE) containing acid phosphatase activity, a common foodstuff which appears to be of low allergenicity. All test proteins were clearly immunogenic when administered by gavage to BN rats, with measurable, and in some cases very vigorous, IgG antibody responses recorded for all animals. Identical exposure of BALB/c strain mice also stimulated detectable IgG antibody responses, with particularly high titers recorded following treatment with peanut agglutinin and somewhat less vigorous responses induced by OVA and PPE. Despite these high titer IgG antibody responses, however, none of the proteins provoked detectable IgE antibody following gavage administration to BN rats. In contrast, in BALB/c mice oral exposure to peanut agglutinin elicited high titer IgE antibody, although IgE antibody responses to both OVA and PPE were much weaker. Parenteral (i.p.) treatment of BALB/c strain mice with each of the test materials induced relatively high titer IgG antibody and a differential potential to stimulate IgE antibody was observed. High titer IgE responses were provoked by i.p. administration of peanut agglutinin and OVA, whereas PPE stimulated little or no detectable IgE antibody. It would appear, therefore, that while it is possible to elicit robust IgE responses by gavage exposure of BALB/c strain mice to some protein allergens, such as peanut agglutinin, such responses are generally weaker and less consistent than those provoked by i.p. administration. Furthermore, gavage treatment failed to induce detectable IgE responses in the BN rat, suggesting that the ability these animals to mount IgE responses is somewhat variable. Following i.p. administration to BALB/c strain mice, these proteins displayed immunological properties consistent with what is known of their allergenic potential in humans, suggesting that,

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following further evaluation with a wider range of proteins, this method may provide one approach to the identification of potential protein allergens. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Food allergy is an important and widespread disease; the best estimates being that in Europe and the USA approximately 1–2% of the adult population is affected, with the prevalence among children being even greater (Taylor et al., 1989; Anderson, 1996; Hourihane, 1998). Most confirmed food allergies are associated with a relatively limited range of produce (cows' milk, eggs, shellfish, fish, peanuts, tree nuts, soybeans and wheat), although there are many other foods and food products that have been implicated also (Hefle et al., 1996). With the development of transgenic crop plants there is a further stimulus to define relevant methods for safety assessment of novel food products. Of particular interest has been consideration of the approaches available to confirm or otherwise the lack of allergenicity of novel gene products (Astwood et al., 1996; Houben et al., 1997; Taylor, 1997; Kimber et al., 1997, 2000; Wal and Pascal, 1998; Gendel, 1998).

To date, the only systematic consideration of the methods available for assessment of the allergenic potential of food and food proteins deriving from genetically modified crop plants resulted from an analysis performed jointly by the International Life Sciences Institute (ILSI) Allergy and Immunology Institute and the International Food Biotechnology Council (Metcalf et al., 1996). Included in their recommendations were examination, using serological methods, of the antigenic identity of novel proteins with known human allergens, consideration of structural similarities and/or the extent of amino acid sequence homology between known allergens and the test protein and evaluation of protein stability in a simulated gastric fluid (Metcalf et al., 1996). Important as such parameters are in the context of an overall safety assessment, such measurements cannot be regarded as providing an unequivocal or definitive assessment of the potential allergenicity of a novel

protein. As a consequence there has in recent years been a growing interest in the development of animal models that would permit a more direct evaluation of the sensitizing potential of proteins. In this context attention has focused on the measurement of induced antibody responses, and in particular the ability of test proteins to stimulate the production of IgE; the class of antibody known to cause allergic sensitization to proteins and to elicit allergic reactions. For this purpose some investigators have elected to employ the Brown Norway (BN) rat; a strain that has been characterized as mounting strong IgE antibody responses. Although methods employing either oral or intraperitoneal administration of protein combined with exposure to adjuvant have been described (Atkinson and Miller, 1994; Atkinson et al., 1996; Miller et al., 1999), the approach employing BN rats that has attracted most interest is one in which the test protein is delivered by daily gavage over a 42 day period in the absence of adjuvant. During and following exposure, specific IgE (and IgG) antibody responses are measured (Knippels et al., 1998a,b, 1999a,b). Using this approach the authors have reported the successful elicitation of IgE antibody responses to ovalbumin (OVA), a known protein allergen (Knippels et al., 1998b).

We have adopted an alternative strategy in which antibody responses are monitored following parenteral administration of test proteins to BALB/c mice; this strain being comparable to BN rats insofar as IgE production is favoured (Hilton et al., 1994, 1997; Kimber et al., 1999; Dearman et al., 2000). In common with the BN rat model described above, it has been possible to generate IgE antibody responses to OVA and to other known protein allergens. Both approaches show some promise and it is possible that there may be some benefit, in certain circumstances, in using both methods in tandem. However, to facilitate design of appropriate testing strategies it is neces-

sary to have a more detailed appreciation of the reliability, sensitivity and selectivity of the various approaches available. As a first step in facilitating this we have in the present investigations compared antibody (IgG and IgE) responses to proteins induced in BN rats by gavage administration and in BALB/c mice following either i.p. or gavage exposure. The test proteins selected for this purpose comprised peanut agglutinin, an allergenic constituent of peanuts, IgE antibodies to which are reported in 20–50% of peanut-allergic individuals (Burks et al., 1994); OVA, *Gal d 1*, a major allergen of chicken egg white (Metcalf, 1985) and a crude potato protein extract (PPE) containing acid phosphatase activity, a common foodstuff for which there is little evidence from the clinic or from experimental studies to suggest is a significant cause of sensitization (Jeannet-Peter et al., 1999).

2. Materials and methods

2.1. Animals

Young adult (8–12 weeks old) female BALB/c strain mice and young adult (8–10 weeks old) female BN rats (Harlan Seralab, Oxfordshire, UK) were used throughout these studies. Food and water were available ad libitum. The diet used was Special Diet Services Rat and Mouse No 1 Maintenance Diet comprising primarily cereal products (Special Diets Services, Witham, Essex). The ambient temperature was maintained at 21 ± 2 °C and relative humidity was $55 \pm 10\%$ with a 12-h light:12-h dark cycle. All experiments were carried out under the provisions of the UK Animals (Scientific Procedures) Act, 1986.

2.2. Test materials

Peanut agglutinin (*Arachis hypogaea*; 99%) and ovalbumin (OVA; Grade V, 98%) were obtained from Sigma Chemical Co, St Louis, MO. A crude potato protein extract (PPE) containing type II acid phosphatase enzymatic activity was also supplied by Sigma, this material was approximately

40% protein (the remainder being primarily salts) and SDS polyacrylamide gel analysis (data not shown) revealed the presence of several bands, indicating a mixture of proteins.

2.3. Sensitization procedures

2.3.1. Oral exposure

Groups of rats or mice ($n = 6$) received a daily dose by gavage of 1 mg of protein in 1 or 0.25 ml of water, respectively, using a blunt ended stainless steel animal feeding needle. Control animals received an equal volume of water alone. Mice were exsanguinated 28 or 42 days after the initiation of exposure. Rats were bled from the tail vein prior to exposure and at weekly intervals thereafter (days 7, 14, 21, 28, and 35) until exsanguination by cardiac puncture 42 days after the initiation of exposure. Individual and pooled serum samples were prepared and stored at -20 °C until analysis.

2.3.2. Intraperitoneal exposure

Groups of mice ($n = 5$) received 0.25 ml of 0.2% peanut lectin, 2% OVA or 10% PPE in phosphate buffered saline (PBS) by i.p. injection. Seven days later treatment was repeated. Fourteen, 28 or 42 days following the initiation of exposure, mice were exsanguinated by cardiac puncture. Individual and pooled serum samples were prepared and stored at -20 °C until analysis.

2.4. Mouse anti-protein IgG antibody analysis

Protein-specific IgG antibodies were detected using enzyme-linked immunosorbent assay (ELISA). Plastic microtiter plates (Nunc, Copenhagen, Denmark) were coated with 100 µg/ml of protein in PBS by overnight incubation at 4 °C. The plates were blocked by incubation for a further 30 min at 37 °C with 2% bovine serum albumin (BSA; Sigma) in PBS. Doubling dilutions of mouse serum samples (derived from individual animals or pooled for each experimental group; starting dilution 1:50) diluted in 0.1% BSA in PBS were added to consecutive wells and incubated for 3 h at 4 °C. There followed a further incubation for 2 h at 4 °C with peroxidase-labelled goat

anti-mouse IgG (Nordic Immunology, Tilberg, The Netherlands) diluted 1:8000 with 1% BSA in PBS. Enzyme substrate (*o*-phenylenediamine and urea hydrogen peroxide) was added and the reaction stopped after 15 min by the addition of 0.5 M citric acid. Between each incubation, the plates were washed with PBS containing 0.05% Tween 20. Substrate conversion was measured as optical density at 450 nm using an automated reader (Flow Laboratories, Irvine, UK). Antibody titers are expressed as the highest dilution at which substrate conversion (OD₄₅₀ nm) was greater than three times background (reagent blank) levels. Where sets of serum samples are compared directly, ELISA analyses were performed on the same day for all of the samples.

2.5. Rat anti-protein IgG antibody analysis

Protein-specific IgG antibodies were detected using ELISA. Plastic microtiter plates (Nunc) were coated with 100 µg/ml of protein in PBS by overnight incubation at 4 °C. The plates were blocked by incubation for a further 30 min at 37 °C with 2% BSA in PBS. Doubling dilutions of rat serum samples (derived from individual animals or pooled for each experimental group; starting dilution 1:50 or 1:25, respectively) diluted in 1% BSA in PBS were added to consecutive wells and incubated for 3 h at 4 °C. There followed a further incubation for 2 h at 4 °C with peroxidase-labelled goat anti-rat IgG (Serotec, Kidlington, UK) diluted 1:2000 with 1% BSA in PBS. Enzyme substrate (*o*-phenylenediamine and urea hydrogen peroxide) was added and the reaction stopped after 15 min by the addition of 0.5 M citric acid. Between each incubation, the plates were washed with PBS containing 0.05% Tween 20. Substrate conversion was measured as optical density at 450 nm using an automated reader (Flow Laboratories). Antibody titers are expressed as the highest dilution at which substrate conversion (OD₄₅₀ nm) was greater than three times background (reagent blank) levels. Where sets of serum samples are compared directly, ELISA analyses were performed on the same day for all of the samples.

2.6. Measurement of mouse anti-protein IgE antibody

The presence in mouse serum of IgE antibodies was detected by homologous passive cutaneous anaphylaxis (PCA) assay. Individual serum samples (neat) were injected (30 µl) into the dermis of the ears of naïve recipient mice ($n = 2$). In some experiments, IgE titer was determined using pooled serum samples diluted to various extents with physiological saline injected (30 µl) into the dermis of the ears of naïve recipient mice ($n = 4$). Concurrent negative control serum samples (derived from naïve animals) diluted to an equivalent extent with physiological saline were incorporated into every PCA assay. Two days later, 0.25 mg of relevant protein, together with Evans Blue dye (1.25 mg), in 0.25 ml of physiological saline were injected intravenously. Thirty min following challenge, mice were killed and the diameter of cutaneous reactions measured. A positive (IgE) response was recorded if the challenge resulted in $a > 3$ mm blue lesion in the skin in the majority of recipient animals (pooled serum samples), with antibody titer recorded as the highest dilution of serum resulting in a positive PCA reaction. For individual serum samples, the mean diameter of the cutaneous reaction in the two recipient animals was recorded, and the serum sample was identified as positive for IgE antibody if the mean diameter of the challenge-induced cutaneous reaction was > 3 mm.

2.7. Measurement of rat anti-protein IgE antibody

The presence in rat serum of IgE antibodies was detected by homologous PCA assay. Individual serum samples (neat) were injected (100 µl) intradermally into the shaved flanks of naïve recipient BN rats. Sixty six h later, 2.5 mg of relevant protein, together with Evans Blue dye (10 mg), in 1 ml of physiological saline was injected intravenously. Thirty min following challenge, rats were killed and the diameter of cutaneous reactions measured. A positive (IgE) response was recorded if the challenge resulted in $a > 5$ mm blue lesion in the skin. Positive control sera

derived from animals exposed systemically (intraperitoneally) to protein and negative control sera from rats exposed by gavage to water only were assayed concurrently.

3. Results

3.1. Antibody responses induced by gavage administration in rats

The kinetics of IgG and IgE antibody responses induced in Brown Norway rats by oral exposure to various proteins was investigated. Animals were exposed to the known food allergens peanut agglutinin or OVA, or to a potato protein extract, a foodstuff which is rarely associated with allergy. Rats ($n = 6$ per group) received 1 mg of protein in 1 ml of water by gavage daily for 42 days according to the protocol of Knippels et al. (1998b). Prior to initiation of exposure, and at weekly intervals thereafter, blood samples were collected and serum samples prepared. Serum samples pooled on an experimental group basis were analyzed for IgG content by ELISA (Fig. 1). Administration of peanut agglutinin resulted in the very rapid induction of IgG antibody, with maximal responses provoked within 21 days of initiation of exposure and detectable IgG antibody observed within 7 days of treatment. Oral exposure to OVA also stimulated the production of IgG antibody, although, the kinetics were somewhat delayed compared with the responses provoked by peanut agglutinin, with IgG antibody not detectable until 14 days after the start of treatment and maximal responses observed after 28 days. A similar kinetic profile was observed for the elicitation of anti-PPE IgG antibody production, with maximal IgG antibody responses induced after 35 days, and antibody first detectable after 14 days of daily gavage.

In subsequent analyses, in order to investigate inter-animal variation in antibody responses, IgG and IgE antibody production was measured in individual serum samples derived 28 and 42 days following the start of treatment (Table 1). Gavage treatment with peanut agglutinin resulted in a vigorous IgG antibody response in all animals.

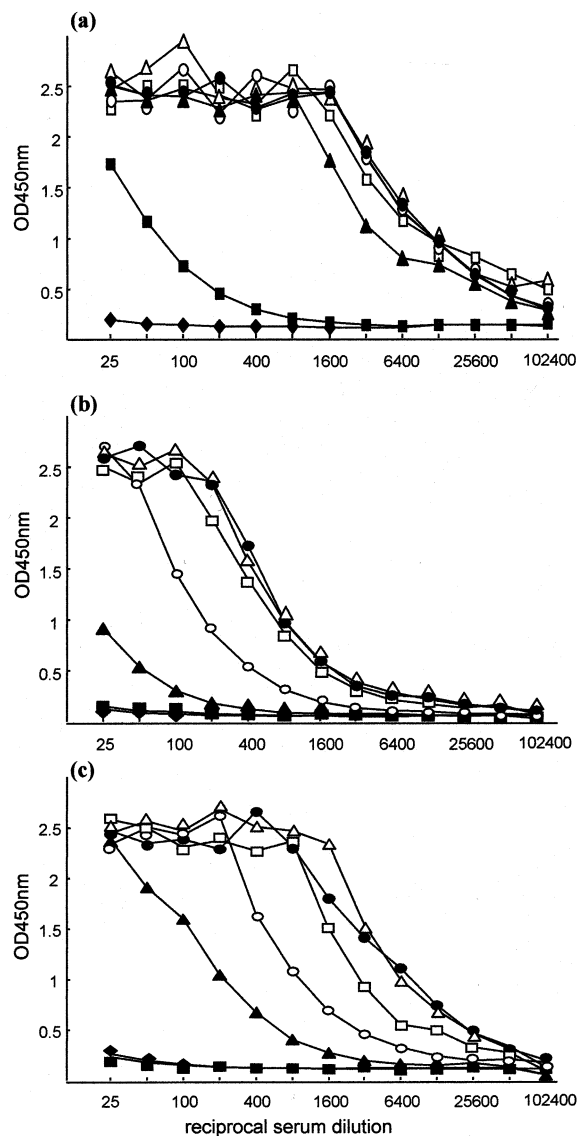


Fig. 1. IgG antibody production following oral exposure of BN rats to peanut agglutinin, OVA or PPE: a kinetic analysis. Groups of rats ($n = 6$) received 1 ml of water containing 1 mg of peanut agglutinin (a), OVA (b) or PPE (c) by gavage daily for 42 days. Animals were bled from the tail vein prior to exposure (day 0; ◆) and bled at weekly intervals thereafter (day 7, ■; day 14, ▲; day 21, ○; day 28, □; day 35, ●) until exsanguination by cardiac puncture 42 days (Δ) after the initiation of exposure. Pooled sera were tested for protein-specific IgG antibody by ELISA.

Table 1

IgG and IgE antibody production following oral exposure of rats to peanut agglutinin, OVA or PPE: inter-animal variation

Reciprocal IgG antibody titer							Number of responders	
							IgG	IgE
<i>Peanut agglutinin</i>								
Animal number	1	2	3	4	5	6		
Day 28	25 600	409 600	51 200	25 600	102 400	51 200	6/6	0/6
Day 42	25 600	204 800	102 400	25 600	51 200	102 400	6/6	0/6
<i>OVA</i>								
Animal number	1	2	3	4	5	6		
Day 28	ND	ND	800	102 400	3200	50	4/4	0/6
Day 42	100	51 200	1600	51 200	3200	1600	6/6	0/6
<i>PPE</i>								
Animal number	1	2	3	4	5	6		
Day 28	25 600	25 600	ND	< 50	200	ND	3/4	0/6
Day 42	819 200	102 400	3200	400	102 400	819 200	6/6	0/6

Groups of rats ($n = 6$) received 1 mg of peanut agglutinin, OVA or PPE in 1 ml of water daily by gavage. Twenty eight or 42 days after the initiation of treatment, blood was sampled and individual sera were tested for the presence of specific IgG antibody by ELISA and IgE antibody by PCA. IgG titer is recorded as the highest dilution at which substrate conversion (OD450 nm) was greater than three times background (reagent blank) levels. Control serum samples (from day 0 prior to treatment) were routinely negative (titers of < 1 in 50) in all IgG ELISAs. All samples were tested for IgE content by PCA using neat serum and were found to be negative. ND = not done.

Consistent with the results with pooled serum samples (cf Fig. 1), maximal antibody responses were observed within 28 days following initiation of exposure, with titers for individual rats at days 28 and 42 being identical, or varying only by one serial dilution. IgG antibody responses induced by administration of OVA showed a marked degree of inter-animal variation, although for the majority of rats, maximal responses were provoked within 28 days of initiation of treatment. A similar level of inter-animal variation was observed in rats treated with PPE. In general, and consistent with the results derived with pooled serum samples (cf Fig. 1), maximal responses to PPE were not achieved within 28 days. Indeed, for one individual rat, IgG antibody titer increased from 1 in 200 to 1 in 102 400 from 28 to 42 days of treatment. Although administration of OVA and PPE resulted in a large degree of inter-animal variation in IgG titer, by 42 days all animals were IgG antibody positive. Oral treatment with all of the three proteins, however, failed to elicit detectable IgE antibody in any animal at any time

point investigated, despite the very high IgG antibody responses stimulated in most animals, particularly those exposed to peanut agglutinin. The failure to detect specific IgE antibody in serum derived from orally exposed animals was not due to a lack of integrity of the rat PCA assay for IgE, as serum samples from animals treated systemically (intraperitoneally) with protein were positive in this assay.

3.2. Antibody responses induced by gavage in mice

The ability of these same three proteins to induce antibody responses in BALB/c strain mice following oral administration was investigated. Animals ($n = 6$) received 1 mg of protein daily by gavage. Twenty eight and 42 days after the initiation of treatment, mice were exsanguinated and individual serum samples examined for IgG antibody expression by ELISA (Fig. 2, Table 2). To ensure integrity and consistency of the IgG assays, concurrent positive control serum samples derived

from intraperitoneally treated animals were incorporated into each ELISA. Treatment of mice with peanut agglutinin resulted in vigorous IgG antibody responses in all animals at both time points measured with equivalent titers to those induced following i.p. exposure (Table 2). In contrast, gavage administration of OVA resulted in very

variable IgG antibody responses (Fig. 2 Table 2), with only a minority of animals responding to OVA with IgG titers similar to those provoked by systemic exposure. A similar degree of variation in serum IgG antibody production was observed following exposure to PPE. After 28 days of daily gavage treatment, only four out of six serum

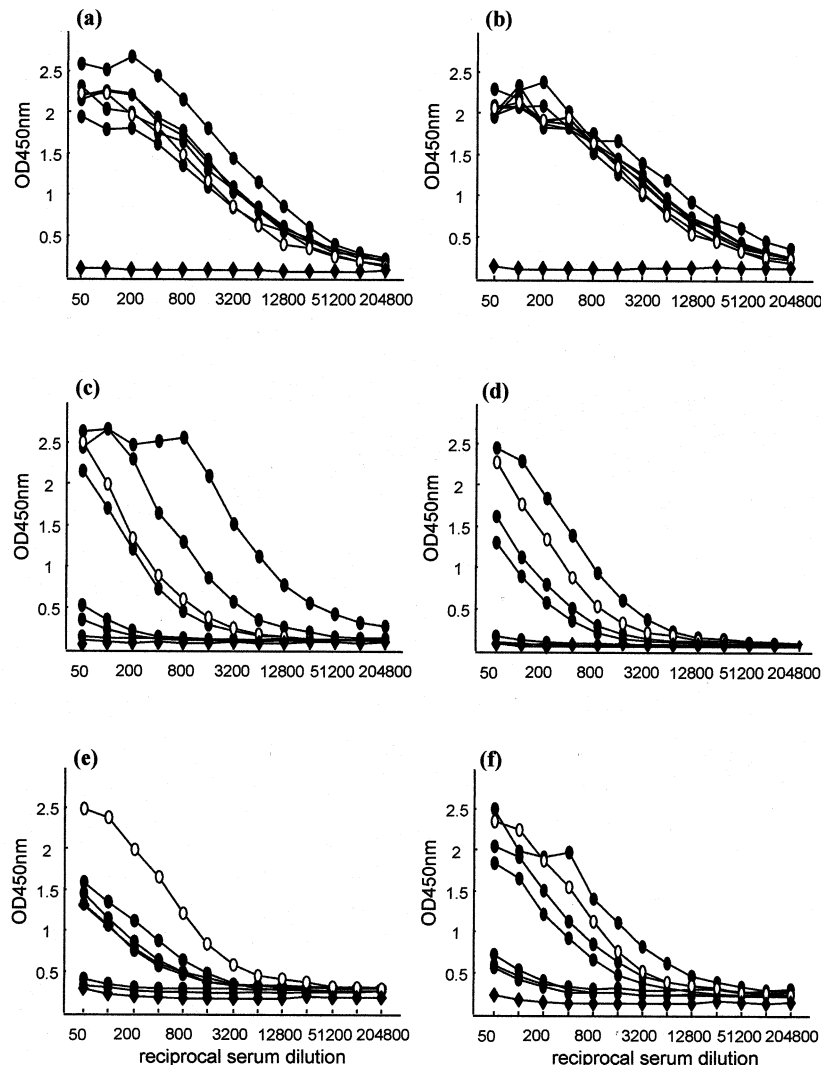


Fig. 2. IgG antibody production following oral exposure of BALB/c strain mice to peanut agglutinin, OVA or PPE: inter-animal variation. Groups of mice ($n = 6$) received 0.25 ml of water containing 1 mg of peanut agglutinin (a, b), OVA (c, d) or PPE (e, f) by gavage daily. Twenty eight (a, c, e) or 42 days (b, d, f) after the initiation of exposure, mice were exsanguinated and individual serum samples tested for protein-specific IgG antibody by ELISA. Each marker (●) represents an individual serum sample derived after gavage administration of protein, with the positive control serum (isolated following intraperitoneal exposure) represented by (○) and negative control serum derived from naïve untreated mice represented by (■).

Table 2

Inter-animal variation in IgG antibody responses following oral exposure of BALB/c strain mice to peanut agglutinin, OVA or PPE

	Reciprocal IgG antibody titer						Number of responders
<i>Peanut agglutinin</i>							
Animal number	1	2	3	4	5	6	
Day 28	51 200	25 600	51 200	25 600	25 600	ND	5/5
Day 42	12 800	25 600	25 600	51 200	102 400	25 600	6/6
<i>OVA</i>							
Animal number	1	2	3	4	5	6	
Day 28	51 200	50	800	< 50	100	3200	5/6
Day 42	50	800	< 50	6400	< 50	800	4/6
<i>PPE</i>							
Animal number	1	2	3	4	5	6	
Day 28	400	< 50	100	100	< 50	100	4/6
Day 42	3200	400	< 50	< 50	< 50	800	3/6

Groups of mice ($n = 6$) received 1 mg of peanut agglutinin, OVA or PPE in 0.25 ml of water daily by gavage. Twenty eight or 42 days after the initiation of treatment, animals were exsanguinated and individual sera were tested for the presence of specific IgG antibody by ELISA. IgG titer is recorded as the highest dilution at which substrate conversion (OD450 nm) was greater than three times background (reagent blank) levels. Negative control serum samples (from naïve animals) were routinely negative (titers of < 1 in 50) in all IgG ELISAs. ND = not done.

samples were positive for IgG, and these had relatively low titers compared with those induced by intraperitoneal exposure. Forty two days following the initiation of exposure, a similar proportion of animals (three out of six) were IgG negative, although the IgG titers observed for the remaining three mice were somewhat higher. Serum isolated from untreated (naïve) mice were invariably negative for IgG (titers of < 1 in 50) in all ELISA assays.

Individual serum samples from orally-exposed mice were examined for IgE content by PCA assay (Fig. 3). Animals which had received 28 or 42 daily treatments with peanut agglutinin and displayed vigorous IgG antibody responses were also all positive for IgE antibody. Exposure by gavage to OVA, in contrast, stimulated the production of IgE in a minority of animals, with only two out of six and one out six mice PCA positive after 28 and 42 daily doses, respectively. Twenty eight days of gavage administration of PPE failed to stimulate detectable IgE antibody in any of the exposed animals. After 42 doses, however, two out of six serum samples were IgE positive.

3.3. Influence of route of exposure on antibody responses induced in mice by proteins

IgG and IgE antibody responses provoked following systemic (i.p.) administration to BALB/c mice of protein were compared with those stimulated by gavage treatment. Animals ($n = 5$) received 0.25 ml of 0.2% peanut agglutinin, 2% OVA or 10% PPE in PBS by intraperitoneal injection on days 0 and 7. Fourteen, 28 and 42 days after the initiation of exposure, individual serum samples were analyzed for the presence of IgG antibody by ELISA (Table 3) and for IgE antibody by PCA assay (Fig. 3). Systemic administration of peanut agglutinin induced high titer IgG antibody production in all exposed mice with the exception of a single animal exsanguinated 28 days after the initiation of treatment which failed to express detectable IgG antibody. Intraperitoneal exposure of mice to OVA or PPE stimulated IgG antibody responses which were generally of higher titer than those observed following gavage administration, although titers showed considerable inter-animal variation. For

all three proteins, maximal IgG responses tended to be provoked 14 days following initiation of systemic exposure, with reduced titers observed after 28 and 42 days.

As observed for administration by gavage (Fig. 3), i.p. treatment with peanut agglutinin resulted in IgE antibody responses in all animals at every time point investigated, with the exception of a

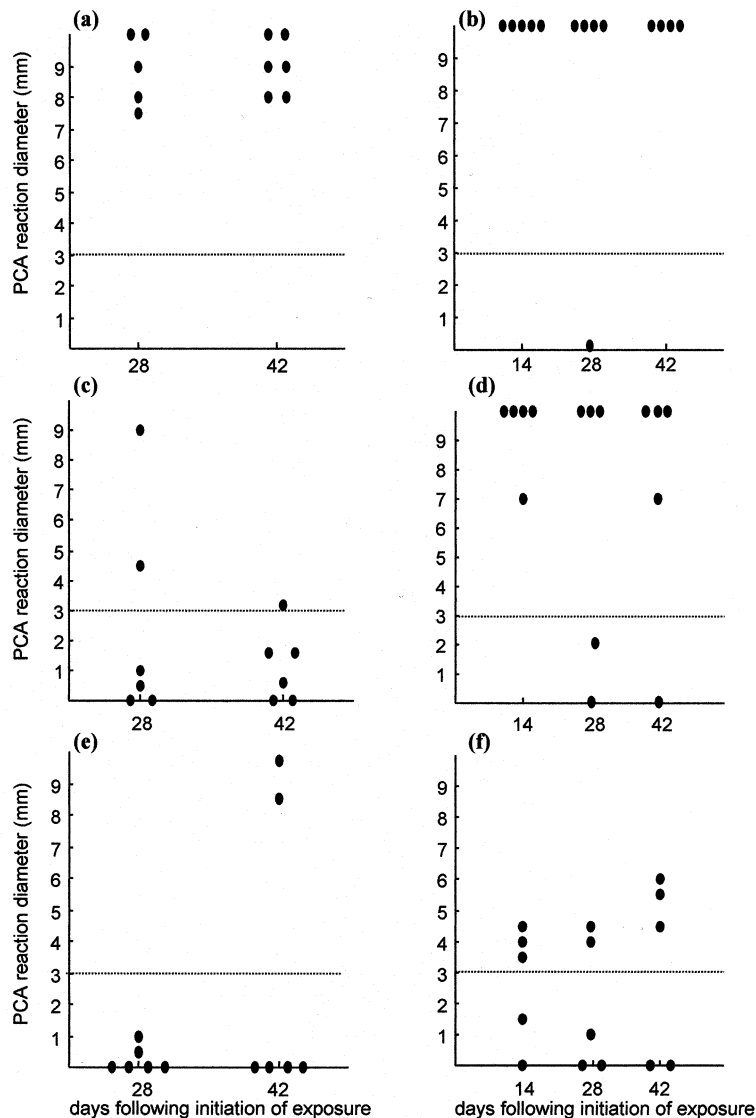


Fig. 3. Influence of route of exposure on IgE antibody production in BALB/c strain mice to proteins. Groups of mice ($n = 6$) received 0.25 ml of water containing 1 mg of peanut agglutinin (a), OVA (c) or PPE (e) by gavage daily. Alternatively, animals ($n = 5$) received 0.25 ml of 0.2% peanut agglutinin (b), 2% OVA (d), or 10% PPE (f) in PBS by intraperitoneal injection with treatment repeated 7 days later. Fourteen, 28 or 42 days after the initiation of exposure, mice were exsanguinated and individual serum samples tested for protein-specific IgE antibody by PCA assay. Each marker (●) represents the mean diameter of the cutaneous reaction in the two recipient mice for an individual serum sample. In every PCA assay, pooled serum isolated from untreated (naïve) animals failed to provoke positive PCA reactions (data not shown). A dashed line represents the criterion for positivity (mean diameter of the challenge-induced cutaneous reaction > 3 mm).

Table 3

Inter-animal variation in IgG antibody responses following systemic (intraperitoneal) exposure of BALB/c strain mice to peanut agglutinin, OVA or PPE: a kinetic analysis

	Reciprocal IgG antibody titer					Number of responders
<i>Peanut agglutinin</i>						
Animal number	1	2	3	4	5	
Day 14	12 800	25 600	6400	6400	12 800	5/5
Day 28	12 800	51 200	12 800	<50	12 800	4/5
Day 42	12 800	6400	3200	3200	3200	5/5
<i>OVA</i>						
Animal number	1	2	3	4	5	
Day 14	12 800	25 600	51 200	409 600	51 200	5/5
Day 28	800	102 400	12 800	12 800	800	5/5
Day 42	102 400	25 600	3200	51 200	25 600	5/5
<i>PPE</i>						
Animal number	1	2	3	4	5	
Day 14	6400	25 600	51 200	3200	12 800	5/5
Day 28	1600	3200	6400	1600	1600	5/5
Day 42	400	1600	3200	3200	12 800	5/5

Groups of mice ($n = 5$) received 0.25 ml of 0.2% peanut agglutinin, 2% OVA or 10% PPE in PBS by intraperitoneal injection on days 0 and 7. Fourteen, 28 or 42 days after the initiation of treatment, animals were exsanguinated and individual sera were tested for the presence of specific IgG antibody by ELISA. IgG titer is recorded as the highest dilution at which substrate conversion (OD450 nm) was greater than three times background (reagent blank) levels. Negative control serum samples (from naïve animals) were routinely negative (titers of <1 in 50) in all IgG ELISAs. ND = not done.

single IgE sero-negative animal at day 28; this animal also failed to express detectable IgG antibody. In contrast to the low frequency of IgE antibody responses stimulated by gavage treatment, systemic exposure to OVA provoked vigorous IgE antibody production in the majority of animals. Administration of PPE by the oral route failed to provoke detectable IgE responses in the majority of animals, with marked IgE antibody induced in only two out of six animals after 42 daily doses of protein. Intraperitoneal treatment stimulated low grade IgE antibody responses at all time points investigated in some animals.

Finally, pooled serum samples from orally and systemically exposed mice were examined for IgE content (Table 4). Consistent with the observations that the majority of individual serum samples isolated following exposure to peanut agglutinin by both routes of exposure and at all time points investigated were IgE positive, high titer IgE responses (one in 32 or greater) in pooled serum samples were recorded also. Oral administration of OVA induced only low titer IgE antibody after 28

daily doses (positive with neat serum), whereas i.p. exposure to OVA stimulated relatively high titer responses, with maximal responses (one in 32) elicited 14 days after initiation of treatment. PPE failed to provoke marked IgE responses at any time point investigated via either route of exposure, with low titer (positive with neat serum) IgE stimulated at only 28 or 42 days after the initiation of i.p. or oral treatment, respectively.

4. Discussion

The data presented here provide the first systematic comparisons of species following oral exposure, and of oral and parenteral routes of exposure in mice, for the assessment of potential protein allergenicity as a function of humoral immune responses. Although, it must be acknowledged that these comparisons are not exhaustive, or sufficiently comprehensive to make detailed recommendations regarding the most appropriate approaches to food allergy safety assessment, it is possible make some important remarks.

The first general point to make is that irrespective of the approach taken (including proposed animal models or considerations of stability or homology with known allergens), hazard identification is predicated on the supposition that protein allergens have structural and/or biological properties that confer on them the potential to induce allergic sensitization. The assumption is that protein allergens have characteristics that distinguish them from other proteins which, although being antigenically different from the recipient (and, therefore, immunogenic), fail to provoke the quality of immune response necessary to cause allergic sensitization. This supposition appears reasonable and among the factors that might influence potential allergenicity are the size and physicochemical properties of the protein, glycosylation status, biologic and enzymatic activity and the way in which the protein is processed and presented to the immune system (Taylor et al., 1987; Bufe, 1998; Maleki et al., 2000; Akdis and Blaser, 2000; Huby et al., 2000). On this basis the strategy of identifying protein allergens as a function of their ability to provoke IgE antibody responses appears justified, although as yet of unconfirmed utility in practice. Against this background, the purpose of the investigations described here was to compare some of the experimental approaches available for allergenicity safety assessments based on induced IgE antibody responses. Excluded from consideration were those methods which incorporate the use of

adjuvant. It is claimed that some adjuvants, including for instance carrageenan and cholera toxin, selectively promote Th2-type cell activation and IgE antibody responses (Nicklin et al., 1985, 1988; Wilson et al., 1989; Marinaro et al., 1995), and a number of animal models have made use of such materials to induce or augment IgE production in response to antigen exposure (Atkinson and Miller, 1994; Atkinson et al., 1996; Miller et al., 1999; Li et al., 1999, 2000). Our view currently is that the use of adjuvant may bias induced responses such as to confer on otherwise non-allergenic proteins the ability to provoke IgE antibody production. As this represents a potential source of false positives in hazard identification attention focused exclusively on those methods that do not require adjuvant.

Consistent with our earlier investigations (Hilton et al., 1994, 1997; Kimber et al., 1999; Dearman et al., 2000) we found that parenteral (intraperitoneal) exposure of BALB/c strain mice to known protein allergens was associated with IgE (and IgG) antibody production. Thus, exposure of mice via this route to peanut agglutinin or OVA in each case resulted in relatively high titer IgE antibody responses. Also in accord with earlier studies was the observation that using this approach it is possible to differentiate, at least in quantitative terms, between proteins of differing allergenic potential. Intraperitoneal injection of mice with PPE (a food material for which there exists little evidence for allergenicity and is as-

Table 4

IgE antibody production following oral or intraperitoneal exposure to peanut agglutinin, OVA or PPE; a kinetic analysis

Reciprocal IgE antibody titer									
Route of exposure	Peanut agglutinin			OVA			PPE		
	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42
Oral	ND	32	32	ND	1	–	ND	–	1
Intraperitoneal	128	32	32	32	16	16	–	1	–

Groups of mice ($n = 6$) received 1 mg of peanut agglutinin, OVA or PPE in 0.25 ml of water daily by gavage. Additional groups of mice ($n = 5$) received 0.25 ml of 0.2% peanut agglutinin, 2% OVA or 10% PPE in PBS via intraperitoneal injection with treatment repeated 7 days later. Fourteen, 28 or 42 days after the initiation of treatment, mice were exsanguinated and pooled sera were tested for the presence of specific IgE antibody by PCA. IgE antibody titer is recorded as the highest dilution of serum resulting in a positive PCA reaction. A negative PCA reaction with neat serum is represented by a dash. In every PCA assay, pooled serum isolated from untreated (naïve) animals failed to provoke positive PCA reactions (data not shown). ND = not done.

sumed to have at most only a modest potential for sensitization), under conditions where this protein was able to stimulate IgG antibody production, was associated with considerably weaker IgE responses and with only a proportion of exposed animals responding. On the basis of earlier investigations it is assumed that such differences between proteins are attributable largely to the quality of immune response that is preferentially induced, with those proteins that provoke vigorous IgE antibody production causing the selective development of Th2-type cells (Dearman et al., 2000).

It has been reported earlier that IgE antibody responses can be induced in BN strain rats following continued oral exposure to OVA (Knippels et al., 1998b, 1999a,b). In those studies administration was by gavage; the evidence being that dietary exposure (ad libitum in drinking water) to OVA under the same conditions induced no, or only very low frequency, antibody responses (Knippels et al., 1998b). It is presumed that, at least in part, the failure to induce strong humoral responses following dietary exposure is secondary to the development of immunological tolerance (Strobel and Mowat, 1998). For this reason all oral exposures in the current investigations were conducted by gavage using a regime described earlier by Knippels et al. (1998b, 1999a,b).

The results reported here indicate clearly that each of the test proteins when administered by gavage were immunogenic in BN rats inducing IgG antibody responses. Although, with each protein there were some inter-animal variations in IgG titers, by 42 days following the initiation of exposure all rats had mounted measurable, and in some cases very vigorous, responses. However, despite the ability of each of these proteins to induce IgG antibody there was no evidence in any instance for IgE antibody production as determined by homologous PCA assay. The failure to detect specific IgE is not attributable to failure of the PCA assay. Sera deriving from BN rats immunized intraperitoneally with OVA were found to contain specific IgE antibody and were used as positive concurrent controls for all rat PCA studies. These data also indicate that the BN rats used in these investigations are not refractory to IgE antibody responses per se. The conclusion is that in the experiments

reported here BN rats failed to mount specific IgE responses to proteins (including allergens) administered using a standard gavage exposure regime, despite the confirmed immunogenicity of these proteins and the ability of the same rats to produce IgE antibody when antigen is delivered by a parenteral route. This is not necessarily an unexpected finding as the failure of OVA to elicit robust IgE responses when administered by gavage to BN rats using the same protocol has been reported earlier. Knippels et al. (1998b) observed that whereas in most experiments 80% or more of BN rats were IgE responders, in a minority of experiments daily gavage dosing with 1 mg of OVA failed to induce specific IgE antibody in any recipient. Jarrett et al. (1976) reported similar experiences when attempting to immunize rats via the oral route. The reasons for the failure to generate IgE responses in BN rats in the present investigations, and the general variability of such responses, is not clear. Although, in theory, the development of oral tolerance is one possibility, to accommodate the fact that vigorous IgG responses were induced it would have to be argued that such tolerance was partial and selective. Probably the more attractive explanation is that the ability of rats, and possibly BN rats in particular, to mount IgE responses is inherently variable and subject to a number of influences including environmental conditions and/or sub-clinical infection (Kemeny, 1994; Metcalfe et al., 1996). In addition it has been demonstrated that unscheduled dietary pre-exposure of the test animals or of the parental generations to the antigen under investigation will impact on the ability of rats to respond to gavage treatment with the induction of antibody (Knippels et al., 1998a).

In these investigations we chose to examine also humoral immune responses in BALB/c strain mice following gavage administration of the same proteins using an identical protocol. It has been suggested earlier that mice are an inappropriate species for this purpose because it is difficult to elicit significant IgE antibody responses in the absence of suitable adjuvants (Taylor and Lehrer, 1996). Moreover, there has been concern that the development in mice of suitable models of food allergy will be hampered by the innate tendency of this species to acquire immunological tolerance

following oral exposure to proteins (Houben et al., 1997). However, although there is no doubt that mice have been used successfully for the investigation of oral tolerance to proteins, as pointed out recently by Strobel and Mowat (1998) the development of systemic immunological hyporesponsiveness is only one of several possible outcomes that may result from oral exposure to proteins and that the effective induction of tolerance is dependent upon a number of factors. Despite possible concerns about the need for adjuvants and the possibility of tolerance induction the results reported here demonstrate clearly that oral exposure of BALB/c mice by gavage was associated with both IgG and IgE antibody responses. Again there was some inter-animal variation in the magnitude of IgG responses. Peanut agglutinin was found to be highly immunogenic with all exposed mice displaying high titer IgG antibody at 28 and 42 days following the initiation of exposure. Responses to OVA and PPE were somewhat less vigorous with only a proportion of treated animals having detectable IgG responses at both time points. Nevertheless, both proteins were clearly immunogenic in at least the majority of exposed mice. Peanut agglutinin also elicited high titer IgE antibody which was detectable in all animals after either 28 or 42 days of treatment. IgE antibody responses to OVA and PPE were much weaker with only one or two mice per group having detectable levels of antibody. The conclusion is that while it is possible to elicit robust IgE responses by gavage exposure of BALB/c mice to some protein allergens (in this instance with peanut agglutinin), such responses are in general much less robust than those provoked by intraperitoneal administration of the same proteins. Of particular relevance is the fact only weak and variable IgE antibody production was elicited by OVA given by gavage, whereas the same protein when administered parenterally resulted in vigorous IgE responses in most or all animals at each time point examined. It must be assumed that with many proteins which are potential allergens it is going to prove more problematic to induce consistent and robust IgE responses by oral dosing compared with other routes of exposure.

Taken together these data confirm earlier observations that it is possible to characterize the inherent potential of proteins to induce IgE antibody responses following intraperitoneal exposure of BALB/c mice. Although, there is clearly a need to evaluate such responses more rigorously with a wider range of allergenic and non-allergenic proteins, our view presently is that this method provides one viable approach to the identification of potential protein allergens. It is argued of course that a predictive test for food allergenicity should ideally incorporate oral administration to reflect more accurately the relevant route of exposure in humans. While such arguments have some merit, certainly in the context of hazard characterization and risk assessment, for the purposes of identifying hazard and the inherent potential of a protein to induce allergic disease the approach using parenteral exposure of BALB/c mice appears to have some utility, not least because it is possible to rank proteins with respect to the vigor of induced IgE responses. In suggesting that this might represent a useful tool in safety assessment it must be acknowledged that identification of an inherent potential for allergenicity may not necessarily translate into a real risk of allergic disease when the same protein is ingested. However, at least the potential hazard has been identified and at the very least it might be possible to determine which proteins have little or no allergenic potential. Alternatively, it might be possible to use data on inherent allergenic potential in concert with other information available for the test protein, including for instance data relating to stability in a simulated gastric fluid, to develop a more holistic assessment of hazard (Metcalf et al., 1996).

The other option is to employ an oral exposure method. Attention earlier has been focused almost exclusively on the rat for this purpose and some success has been reported, at least with respect to the elicitation of IgE responses to OVA (Knippels et al., 1998b). However, it is apparent that such responses can be variable and in our hands a standard protocol failed to elicit any positive responses. It is premature to conclude that this method is inherently unreliable or insufficiently selective, but clearly it will be necessary for more searching analyses to be conducted before firm

recommendations can be made. Finally, it appears that the BALB/c mouse may provide an alternative to the rat for oral exposure studies, although it is apparent that this approach is less sensitive in terms of IgE responses than is i.p. exposure in the same strain.

Although currently no well validated animal test method is available for protein allergen identification, there is no doubt that continued effort in this area, and the conduct of comparative analyses such as those conducted here, will provide a sound basis for the selection of an appropriate approach that can be used in concert with other data to provide for more thorough food safety assessments.

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