

Evaluation of protein allergenic potential in mice: dose–response analyses

R. J. Dearman*, S. Stone*, H. T. Caddick*, D. A. Basketter† and I. Kimber*

*Syngenta Central Toxicology Laboratory, Macclesfield, Cheshire SK10 4TJ, UK and †Unilever Safety and Environmental Assurance Centre, Sharnbrook, Bedfordshire MK44 1LQ, UK

Summary

Background With the increasing interest in novel foods derived from transgenic crop plants, there is a growing need for the development of approaches for the characterization of the allergenic potential of proteins. Whereas immunogenicity is a common property of foreign proteins, including food proteins, relatively few are significant dietary allergens with the inherent capacity to provoke IgE antibody production and immediate-type hypersensitivity responses.

Objective In order to evaluate an approach for the measurement of the allergenic potential of proteins, detailed dose–response analyses of humoral immune responses induced following systemic exposure of BALB/c strain mice to proteins known to differ in terms of sensitizing activity have been conducted.

Results Mice were exposed to a range of concentrations of ovalbumin, a major allergenic constituent of hen's egg, a purified peanut allergen, *Arachis hypogaea* agglutinin, or to the milk allergen bovine serum albumin, and to materials considered to lack significant allergenicity: a crude potato protein extract and a purified potato protein, potato agglutinin. The specific IgE antibody was measured by homologous passive cutaneous anaphylaxis assay, and the specific IgG antibody was measured by enzyme-linked immunosorbent assay. Each of the five proteins was immunogenic in mice, inducing IgG antibody responses at all doses tested, although there was some variation with respect to the vigour of IgG responses. Marked differences in the capacity of these proteins to induce IgE responses were observed, however, with relatively high-titre IgE antibody provoked by all three allergens over the dose ranges examined, whereas the potato proteins stimulated low-titre IgE antibody at the highest dose (10%) only. Importantly, differences in IgE antibody production have been observed against a background of equivalent immunogenicity (IgG antibody responses).

Conclusion The data presented here suggest that the measurement of antibody (IgE) responses in BALB/c mice appears to identify allergens accurately and to distinguish them from those materials that apparently lack allergenicity.

Keywords protein allergenicity, dose responses, IgE, IgG, systemic exposure, predictive testing

Submitted 10 October 2002; revised 17 May 2003; accepted 22 July 2003

Introduction

With the growing interest in the development of novel foods, including those derived from genetically modified crop plants, there has been considerable debate about the most appropriate strategies for safety assessment. One of the main issues is the need to determine whether the products of novel genes have the potential to induce allergic sensitization [1–3], and this was the focus of a recent British Society for Allergy and Clinical Immunology (BSACI) Working Party [4]. There is no doubt that food allergy in general is an important health issue and it is likely that, in common with other forms of atopic allergic disease, both the incidence and prevalence are increasing [5, 6]. The joint considerations of the International Food Biotechnology Council (IFBC) and the International

Life Sciences Institute (ILSI) Allergy and Immunology Institute resulted in the first systematic approach for the assessment of the allergenic potential of novel foods in the form of a hierarchical decision tree [2]. The strategy incorporated examination of amino acid sequence homology with, and/or structural similarity to, known allergenic proteins, measurement of the stability (digestibility) of the protein in a simulated gastric fluid (SGF) and consideration of the serological identity of the protein of interest with known human allergens [2]. Stability in SGF is measured due to the observation that there is a correlation between the ability of proteins to resist proteolytic digestion and their allergenic potential, with many confirmed human allergens being stable in SGF, whereas common plant proteins that apparently lack allergenic potential are digested very rapidly [7].

Although the investigations described above provide useful information as part of the overall safety assessment of novel food proteins, particularly in those cases where proteins are

Correspondence: RJ Dearman, Syngenta Central Toxicology Laboratory, Macclesfield, Cheshire SK10 4TJ, UK.

Email: rebecca.dearman@syngenta.com

found to be homologous with known human allergens, they do not provide a direct evaluation of the inherent sensitizing potential. For this reason, there has been a growing interest in the development of appropriate animal models. Although the view at the time of the original IFBC/ILSI deliberations was that there were no suitable animal models available, considerable progress has been made in the intervening period. Thus, the special consultation panel convened in 2001 by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) to review food allergenicity testing concluded that with more extensive evaluation, animal models may contribute valuable additional information regarding the allergenic potential of novel foods [8]. Effort has therefore been directed towards characterizing allergenicity as a function of the ability of proteins to provoke immune responses in experimental models, and in particular their ability to induce IgE antibody responses, this being the class of antibody known to be the biological mediator of most food-allergic reactions. Some investigators have chosen to focus on species such as the dog [9] or the swine [10], where allergic sensitization may be monitored as a function of both induced IgE antibody responses (often measured by skin prick testing) and oral challenge-induced manifestations of gastrointestinal symptoms. Others have utilized the Brown Norway (BN) rat, a strain that is predisposed to make strong IgE antibody responses, with protocols employing oral or systemic (intraperitoneal; i.p.) exposure and the presence or absence of adjuvant [11–14].

The approach we have adopted has been to use the BALB/c strain mouse, a strain that is also predisposed to make strong IgE antibody responses. We have focused on the measurement of antibody (IgG and IgE) responses induced following parenteral (i.p.) exposure in the absence of adjuvant [15–17]. We have demonstrated previously that there are marked differences in the ability of ovalbumin (OVA), a relatively common food allergen [18], and bovine serum albumin (BSA), a protein considered to be less allergenic, to induce vigorous IgE antibody responses, despite both materials inducing strong IgG antibody responses [15, 17]. Antibody responses induced following i.p. treatment with OVA have also been compared with those provoked by peanut agglutinin, a constituent of peanuts, to which IgE antibody can be detected in 20–50% of peanut-allergic individuals [19], and a crude potato protein extract (PPE), a common foodstuff for which there is little evidence from the clinic or from experimental studies to suggest significant sensitizing potential [20]. The differential ability of these proteins to induce IgE antibody production was consistent with what is known of their relative sensitizing potential in humans, particularly when the concentrations used were taken into account, with both 0.2% peanut agglutinin and 2% OVA stimulating high-titre IgE antibody, whereas 10% PPE failed to stimulate marked IgE responses [16]. In the current investigations, we have compared IgE and IgG antibody responses stimulated following i.p. exposure of BALB/c strain mice to the allergens peanut agglutinin, OVA and BSA with those provoked by exposure to materials considered to be lacking in allergenicity, a purified potato protein, potato agglutinin and PPE. Dose–response analyses have been conducted to facilitate further comparisons between proteins of differing allergenic potential. The majority of previous

investigations using rodents have examined serological responses induced by proteins at a single exposure concentration or dose responses have been conducted with a single allergenic protein [11, 13, 14, 16, 17, 21]. The ability to induce IgE responses has been examined under conditions where all proteins are clearly immunogenic (eliciting an IgG antibody response).

Materials and methods

Animals

Young adult (8–12 weeks old) female BALB/c strain mice (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, UK). The ambient temperature was maintained at $21 \pm 2^\circ\text{C}$ and the relative humidity was $55 \pm 10\%$ with a 12 h light/dark cycle. All experiments were carried out under the provisions of the UK Animals (Scientific Procedures) Act, 1986.

Test materials

Commercial preparations of OVA (Grade V, 98% pure), BSA (Grade V, 98% pure), peanut (*Arachis hypogea*) agglutinin (98% pure), potato (*Solanum tuberosum*) agglutinin and a crude PPE containing type II acid phosphatase enzymatic activity were obtained from Sigma Chemical Co. (St Louis, MO, USA). Analysis of PPE by Western blotting using a monoclonal antibody directed against patatin (kindly supplied by Dr R. E. Goodman (Monsanto, St Louis, MO, USA), the major allergenic component of potato [20], revealed that this extract contained detectable amounts of this protein (data not shown).

Sensitization procedure

Groups of mice ($n = 5$) received 0.25 mL of various concentrations (w/v) of protein in phosphate-buffered saline (PBS) by i.p. injection. Seven days later the treatment was repeated. Mice were exsanguinated 14 days after the initiation of exposure. Individual and pooled serum samples were prepared and stored at -20°C until analysis.

Anti-protein IgG antibody analysis

Protein-specific IgG antibodies were detected using an ELISA. Plastic microtitre plates (Nunc, Copenhagen, Denmark) were coated with $100 \mu\text{g/mL}$ 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 (Sigma) in PBS (OVA, peanut agglutinin, potato agglutinin or PPE) or with 5% normal goat serum (NGS) in PBS (BSA). Doubling dilutions of mouse serum samples (derived from individual animals; starting dilution 1 : 25) diluted in 1% BSA in PBS (OVA, peanut agglutinin, potato agglutinin or PPE) or with 5% NGS in PBS (BSA) 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 (OVA, peanut agglutinin, potato agglutinin or PPE) or with 5% NGS in PBS (BSA). An enzyme substrate (*o*-phenylenediamine and urea hydrogen peroxide) was added and the reaction was 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 the optical density at 450 nm using an automated reader (Flow Laboratories, Irvine, UK). Antibody titres were calculated as the highest dilution at which substrate conversion (OD 450 nm) was greater than 0.5. For control serum derived from naïve (untreated) animals, OD 450 nm readings never exceeded this value even at the maximum concentration tested. Data are expressed as mean and SE of IgG reciprocal titre (log 2) for each treatment group.

Measurement of anti-protein IgE antibody

The presence of IgE antibodies in mouse serum was detected by a homologous passive cutaneous anaphylaxis (PCA) assay. In order to determine the number of IgE responder animals per treatment group, PCA assays were performed with individual serum samples (neat). Individual serum samples (neat) were injected (30 µL) into the dermis of the ears of naïve recipient mice ($n = 2$). The amount of serum isolated from each animal varied between 150 and 250 µL and, due to the amount of serum required for injection plus the volume required for the dead space of the syringe and the luer lock of the needle, a minimum of approximately 450 µL of serum is required in order to determine the IgE titre. Because of these limitations on the amount of serum available per individual animal, IgE titres were determined using serum samples pooled on a treatment group basis, which in some treatment groups comprised both negative and positive individual serum samples. Pooled serum samples diluted to various extents with physiological saline were 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 minutes following the challenge, mice were killed and the diameter of cutaneous reactions was 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 titre 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 the IgE antibody if the mean diameter of the challenge-induced cutaneous reaction was >3 mm.

Statistical analyses

For IgG antibody responses, the reciprocal IgG titres for the individual serum samples were considered by analysis of variance following logarithmic transformation, separately for each concentration of protein. The differences between

individual group means were compared using Student's *t*-test based on the error mean square of the analysis of variance. The statistical significance of differences in the number of IgE and IgG responder animals in each dose group was considered by comparing each pair of groups using Fisher's exact test. The statistical significance of differences in IgE titres between pairs of proteins was considered by comparing the whole dose response using the Sign test. Statistical analyses were conducted using the Univariate procedure in the Statistical Analysis System (SAS) User's Guide [22].

Results

Dose responses to proteins: number of IgG and IgE antibody responders

The IgG and IgE antibody responses to food proteins of varying allergenic potential were first analysed as a function of the number of responder animals per treatment group. Animals received 0.25 mL of various concentrations of protein by i.p. injection on days 0 and 7. A dose range from 0.2% to 10% protein was administered for OVA, BSA, potato agglutinin and PPE. For peanut agglutinin, previous experiments have demonstrated that using this exposure regimen, the treatment of mice with as little as 0.2% of this protein induces a relatively high-titre IgE antibody (1:128) [16], so for this protein a different concentration range (from 0.01% to 0.2%) was used. Fourteen days after the initiation of exposure, animals were exsanguinated and individual serum samples were prepared. Each serum sample was analysed by ELISA (starting dilution 1:25) to determine the number of IgG responder animals per treatment group, and by homologous PCA assay (neat serum samples) to determine the number of IgE responder animals per treatment group (Table 1: OVA, BSA, potato agglutinin and PPE; Table 3: peanut agglutinin). For each protein examined, negative controls (naïve serum samples) were tested concurrently and were invariably negative by both ELISA and PCA (data not shown). The statistical significance of differences in the number of responder animals per treatment group for a given concentration of protein was assessed using Fisher's exact test. At all doses of each of the four proteins displayed in Table 1 (0.2–10%), all serum samples tested positive for specific IgG by ELISA, and there were no significant differences in the percentage of IgG responder groups between proteins. Exposure to 0.01–0.2% peanut agglutinin also stimulated detectable IgG antibody in all mice in all dose groups (Table 3). Significant differences were observed, however, in the ability of these proteins to induce IgE antibody responses (Table 1: OVA, BSA, potato agglutinin and PPE). At the highest doses of protein administered (5% and 10%), a specific IgE antibody was detected in the majority of serum samples for all four proteins examined, with no significant differences recorded between groups. Treatment with 0.5–2% OVA and BSA also provoked detectable IgE antibody in 75–100% of mice per experimental group, whereas exposure to the same concentrations of potato agglutinin or PPE either failed to elicit measurable IgE antibody or one of five mice only were positive. These differences in the number of IgE responder mice reached

Table 1. Antibody responses following systemic exposure to ovalbumin (OVA), bovine serum albumin (BSA), potato agglutinin or potato protein extract (PPE): number of IgG and IgE responder animals per group and statistical analyses

	10%		5%		2%		1%		0.5%		0.2%	
	IgG	IgE	IgG	IgE	IgG	IgE	IgG	IgE	IgG	IgE	IgG	IgE
<i>No. of responders</i>												
OVA	5/5	4/4	5/5	5/5	4/4	4/4	5/5	5/5	5/5	4/5	5/5	5/5
BSA	5/5	5/5	5/5	4/5	5/5	4/5	5/5	5/5	5/5	3/4	4/4	2/4
Potato agglutinin	5/5	5/5	5/5	2/5	5/5	0/5	4/4	0/4	5/5	0/5	4/4	0/4
PPE	5/5	4/5	5/5	3/5	5/5	1/5	5/5	1/5	5/5	0/5	5/5	0/5
<i>Statistical comparisons</i>												
OVA vs. BSA	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
OVA vs. potato agglutinin	NS	NS	NS	NS	NS	**	NS	**	NS	*	NS	**
OVA vs. PPE	NS	NS	NS	NS	NS	*	NS	*	NS	*	NS	**
BSA vs. potato agglutinin	NS	NS	NS	NS	NS	*	NS	**	NS	*	NS	NS
BSA vs. PPE	NS	NS	NS	NS	NS	NS	NS	*	NS	*	NS	NS
Potato agglutinin vs. PPE	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Groups of mice ($n = 5$) received 0.25 mL of various concentrations (0.2–10%) of OVA, BSA, potato agglutinin or PPE by i.p. injection on days 0 and 7. Seven days later, the animals were exsanguinated and individual serum samples were tested for the presence of specific IgG antibody by ELISA or for the presence of specific IgE antibody by homologous passive cutaneous anaphylaxis (PCA) assay. Negative control serum samples (from naïve animals) were tested concurrently and were routinely negative by ELISA and PCA (data not shown). The number of IgG and IgE responders per treatment group is displayed. NS = not significant; * $P < 0.05$; ** $P < 0.01$ by Fisher's exact test.

statistical significance at the lower doses. At the lowest dose tested (0.2%), all OVA-treated mice were IgE positive, whereas potato agglutinin and PPE again failed to stimulate measurable IgE ($P < 0.01$). Exposure to peanut agglutinin (0.02–0.2%) also induced vigorous IgE responses, with all animals testing positive for IgE, although following treatment with 0.01% peanut agglutinin, only one of five mice was IgE positive (Table 3). For the 0.2% dose group, the only group for which it is possible to perform comparative statistical analyses with the other proteins, differences between peanut agglutinin and potato agglutinin and PPE reached statistical significance ($P < 0.01$).

Dose responses to proteins: IgG and IgE antibody titres

In subsequent analyses, IgG and IgE antibody titres were measured. IgG antibody titres were determined by ELISA using individual serum samples (starting dilution 1:25); the mean and SE are displayed for all five proteins in Fig. 1 and statistical analyses in Table 2 (OVA, BSA, potato agglutinin and PPE). Consistent with the number of IgG responder animals recorded for each treatment group (cf. Tables 1 and 3), i.p. administration of all five proteins resulted in marked IgG antibody responses, with particularly high-titre IgG being recorded following exposure to as little as 0.02% peanut agglutinin (Fig. 1). The dose–response analyses revealed some modest variations in overall immunogenicity among the proteins investigated, with PPE stimulating considerably lower titres of IgG antibody compared with equivalent doses of OVA, BSA and potato agglutinin, such differences reaching statistical significance for all three proteins at 1%, 2%, 5% and 10% (Table 2). However, IgG titres were comparable over most of the dose range for OVA and potato agglutinin, with significant differences in IgG titre recorded only at the lowest concentration tested (0.2%). Very similar IgG titres were also observed following i.p. administration of OVA and BSA, and potato agglutinin and BSA.

Indeed, differences only reached statistical significance at 0.5% and 1%, where BSA IgG titres were somewhat higher than those recorded for OVA and for potato agglutinin, and at 0.2%, where BSA IgG titres were significantly higher than those elicited by potato agglutinin. At the highest concentration of peanut agglutinin tested (0.2%), significantly higher IgG titres were induced than those stimulated by exposure to equivalent amounts of OVA, BSA, potato agglutinin or PPE ($P < 0.01$).

IgE antibody responses were measured by a homologous PCA assay. Because of the limitations in the volume of serum available for these measurements, IgE antibody titres were determined using serum samples pooled on a treatment group basis and are shown for all five proteins in Fig. 2. Consistent with the differences recorded in the percentage of IgE responder animals per treatment group (cf. Tables 1 and 3), marked differences between proteins were observed with respect to induced IgE antibody responses. It is apparent, however, that for some treatment groups, where the majority of animals were recorded as being IgE responders based on individual serum sample analyses, this did not translate into a positive IgE response (titre greater than 1) in the pooled serum sample (cf. PPE at 5%; three out of five IgE responder mice and a titre of <1 for pooled serum). This is a consequence of the fact that the serum pool is derived from IgE responder and non-responder individual serum samples, with, in some instances, the IgE-positive samples being diluted out by the IgE-negative serum samples. The most vigorous IgE antibody responses were recorded following the administration of peanut agglutinin, with relatively high-titre IgE antibody (titres of 1:16 and above) induced by exposure to 0.02–0.2% protein and detectable IgE antibody stimulated even after treatment with 0.01% peanut agglutinin (Table 3). Although these high-titre IgE responses were recorded at doses that were orders of magnitude lower than the dosing range utilized for the other four proteins (0.2–10%), it is not possible to demonstrate formally the statistical significance of

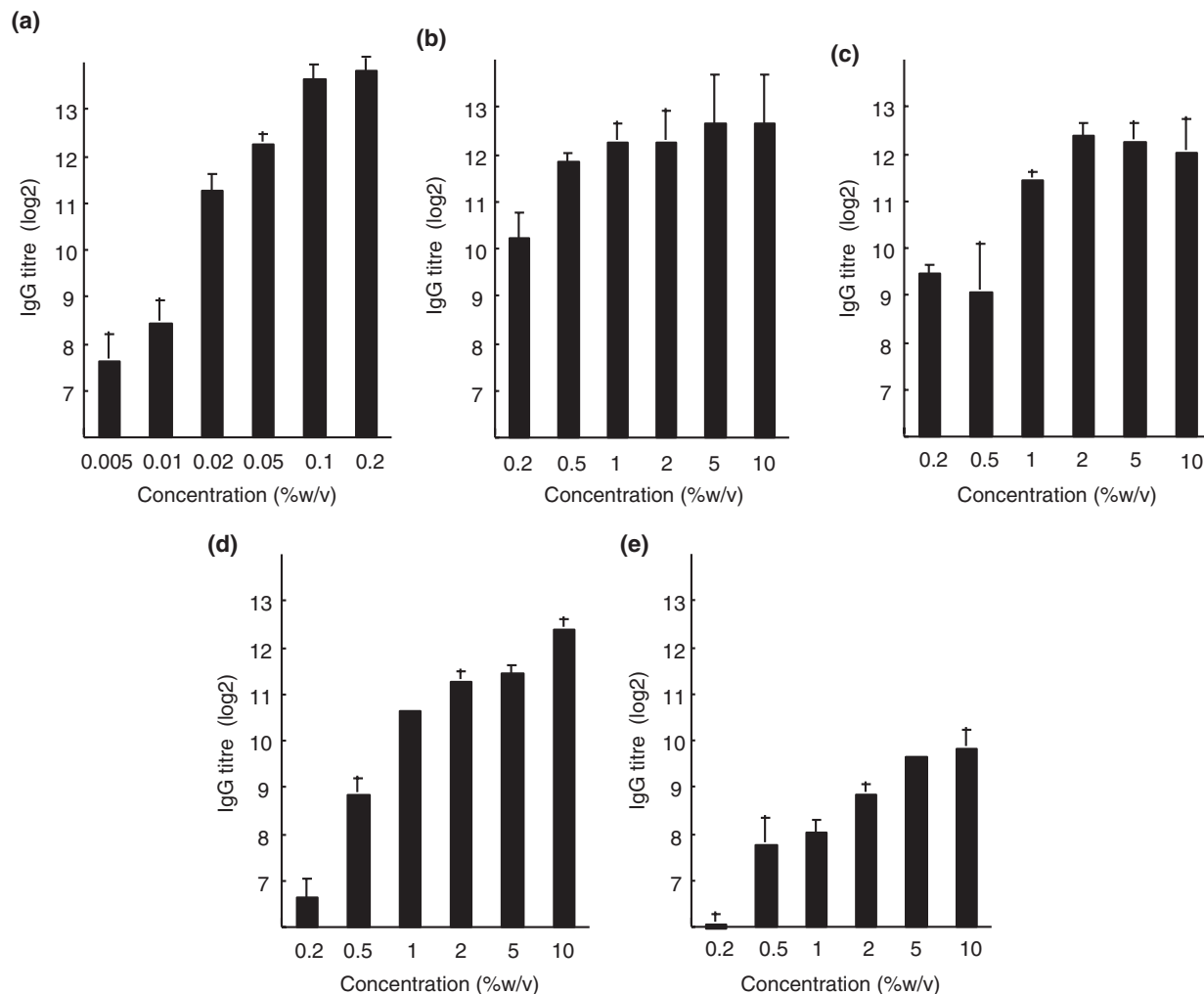


Fig. 1. IgG antibody responses following systemic exposure to peanut agglutinin, ovalbumin (OVA), bovine serum albumin (BSA), potato agglutinin or potato protein extract (PPE). Groups of mice ($n = 5$) received 0.25 mL of various concentrations of peanut agglutinin (a) (0.01–0.2%) or of OVA (b), BSA (c), potato agglutinin (d) or PPE (e) (0.2–10%) by i.p. injection on days 0 and 7. Seven days later, the animals were exsanguinated and individual serum samples were tested for the presence of specific IgG antibody by ELISA. Negative control serum samples (from naïve animals) were tested concurrently and were routinely negative (data not shown). Data are expressed as mean and SE of IgG reciprocal titre (log2) for each treatment group.

these findings. Treatment with OVA and BSA also elicited vigorous IgE antibody responses, with maximal titres of 1:64 and 1:16 recorded, respectively. Interestingly, despite analyses of IgG antibody production revealing that OVA and BSA were of equivalent immunogenicity over most of the dose range used, although BSA stimulated significantly higher IgG titres when dosed at 0.5% and 1%, statistical analyses of IgE responses demonstrated that over this concentration range, OVA displayed a significantly higher potential to provoke IgE antibody responses (Fig. 2; $P < 0.05$). In contrast, compared with peanut agglutinin, OVA and BSA, each of which induced marked IgE antibody responses, i.p. exposure to potato agglutinin and PPE did not result in high-titre IgE production. At the majority of doses tested (0.2–5%), these two proteins failed to elicit detectable IgE antibody, and even at the highest dose tested (10%), only low-titre IgE (1:2 or positive with neat serum) responses were recorded. These IgE responses were significantly lower than those induced by exposure to either OVA or BSA ($P < 0.05$). Of particular importance is the fact that for the comparisons

of potato agglutinin and OVA, for example, these marked differences in IgE antibody production are observed under conditions of equivalent immunogenicity with respect to IgG antibody responses (cf. Table 2).

Discussion

The intrinsic ability of various food proteins to stimulate IgG and IgE antibody responses following systemic (i.p.) administration to the BALB/c strain mouse has been examined. Dose responses have been performed using proteins that are known allergens (peanut agglutinin, OVA and BSA) [18, 19, 23, 24] and materials considered to be lacking in allergenicity: a crude PPE and a purified potato protein, potato agglutinin [20]. All five proteins were immunogenic in mice, inducing IgG antibody responses at all doses tested, although there was some variation with respect to the vigour of IgG responses. Marked differences in the capacity of these proteins to induce IgE responses were observed, however, with relatively

Table 2. IgG antibody responses following systemic exposure to ovalbumin (OVA), bovine serum albumin (BSA), potato agglutinin (PA) or potato protein extract (PPE): titres and statistical analyses

IgG (mean $\pm$ SD) reciprocal titre (log 2)	IgG					
	10%	5%	2%	1%	0.5%	0.2%
OVA mean $\pm$ SD	12.04 $\pm$ 1.52	12.24 $\pm$ 0.89	12.39 $\pm$ 0.50	11.44 $\pm$ 0.45	9.04 $\pm$ 2.30	9.44 $\pm$ 0.45
OVA vs. BSA	NS	NS	NS	*	**	NS
OVA vs. PA	NS	NS	NS	NS	NS	**
OVA vs. PPE	*	**	**	**	NS	**
BSA mean $\pm$ SD	12.64 $\pm$ 2.35	12.6 $\pm$ 2.35	12.2 $\pm$ 1.52	12.2 $\pm$ 0.89	11.8 $\pm$ 0.45	10.2 $\pm$ 1.14
BSA vs. OVA	NS	NS	NS	*	**	NS
BSA vs. PA	NS	NS	NS	**	**	**
BSA vs. PPE	**	**	**	**	**	**
PA mean $\pm$ SD	12.2 $\pm$ 0.55	11.4 $\pm$ 0.45	11.2 $\pm$ 0.55	10.6 $\pm$ 0	8.84 $\pm$ 0.84	6.64 $\pm$ 0.82
PA vs. OVA	NS	NS	NS	NS	NS	**
PA vs. BSA	NS	NS	NS	**	**	**
PA vs. PPE	*	*	**	**	NS	NS
PPE mean $\pm$ SD	9.84 $\pm$ 0.84	9.64 $\pm$ 0	8.84 $\pm$ 0.45	8.04 $\pm$ 0.55	7.44 $\pm$ 1.3	6.04 $\pm$ 0.55
OVA vs. PPE	*	**	**	**	NS	**
BSA vs. PPE	**	**	**	**	**	*
PA vs. PPE	*	*	**	**	NS	NS

Groups of mice ($n = 5$) received 0.25 mL of various concentrations (0.2–10%) of OVA, BSA, PA or PPE by i.p. injection on days 0 and 7. Seven days later, animals were exsanguinated and individual serum samples were tested for the presence of specific IgG antibody by ELISA. Negative control serum samples (from naïve animals) were tested concurrently and were routinely negative (data not shown). The mean and SD IgG titre (reciprocal log 2) for each treatment group is displayed. NS = not significant; * $P < 0.05$; ** $P < 0.01$ by two-sided Student's t -test based on the error mean square of the analysis of variance.

Table 3. IgG and IgE antibody responses following systemic exposure to peanut agglutinin: mean titres and number of IgG and IgE responder animals per group

	Concentration (%w/v)				
	0.2	0.1	0.05	0.02	0.01
IgE titre	128	64	64	16	2
Number of IgE responders	5/5	5/5	5/5	5/5	1/5
IgG titre (mean)	13.84	13.64	12.24	11.24	8.44
SD	1.1	0.71	0.55	0.89	1.1
Number of IgG responders	5/5	5/5	5/5	5/5	5/5

Groups of mice ($n = 5$) received 0.25 mL of various concentrations (0.01–0.2%) of peanut agglutinin in PBS by i.p. injection on days 0 and 7. Seven days later, the animals were exsanguinated and individual serum samples were tested for the presence of specific IgG antibody by ELISA or for the presence of specific IgE antibody by homologous passive cutaneous anaphylaxis (PCA) assay to determine the number of responders per group. Pooled serum samples were analysed by PCA to determine IgE titre on a treatment group basis. Negative control serum samples (from naïve animals) were tested concurrently and were routinely negative by ELISA and PCA (data not shown). The mean and SD IgG titre (reciprocal log 2), mean reciprocal IgE titre and the number of IgG and IgE responders per treatment group are displayed.

high-titre IgE antibody provoked by all three allergens over the dose ranges examined, whereas the potato proteins stimulated low-titre IgE antibody at the highest dose (10%) only. These differences in IgE titre for pooled serum samples were reflected by significant differences in the number of IgE responder animals per treatment group. Importantly, differences in IgE antibody production have been observed against a background of equivalent overall immunogenicity (IgG antibody responses). In these studies, statistical analyses have been applied to both IgG and IgE antibody dose responses. It is useful for the interpretation of these data to be able to determine whether proteins induced IgG antibody responses of equivalent magnitude. However, very distinct differences are recorded in IgE profiles after exposure to peanut agglutinin, OVA and BSA compared with those provoked

by the administration of potato agglutinin and PPE, with the latter proteins provoking detectable (low) titre IgE antibody only at the highest concentration tested, whereas the allergens each stimulated the IgE antibody over the complete dose range used. These differences observed in IgE antibody titres are therefore so marked that it is not necessary to apply statistics in order to conclude that these proteins display very substantial differences in their potential to stimulate IgE. The application of statistics may be more appropriate under conditions where less polarized responses are induced.

Of particular interest is one of the proteins considered here, potato agglutinin, which is believed to lack a significant sensitizing potential in humans, and in these studies failed to stimulate IgE antibody responses following i.p. treatment of the BALB/c strain mice. The fact that this protein, unlike

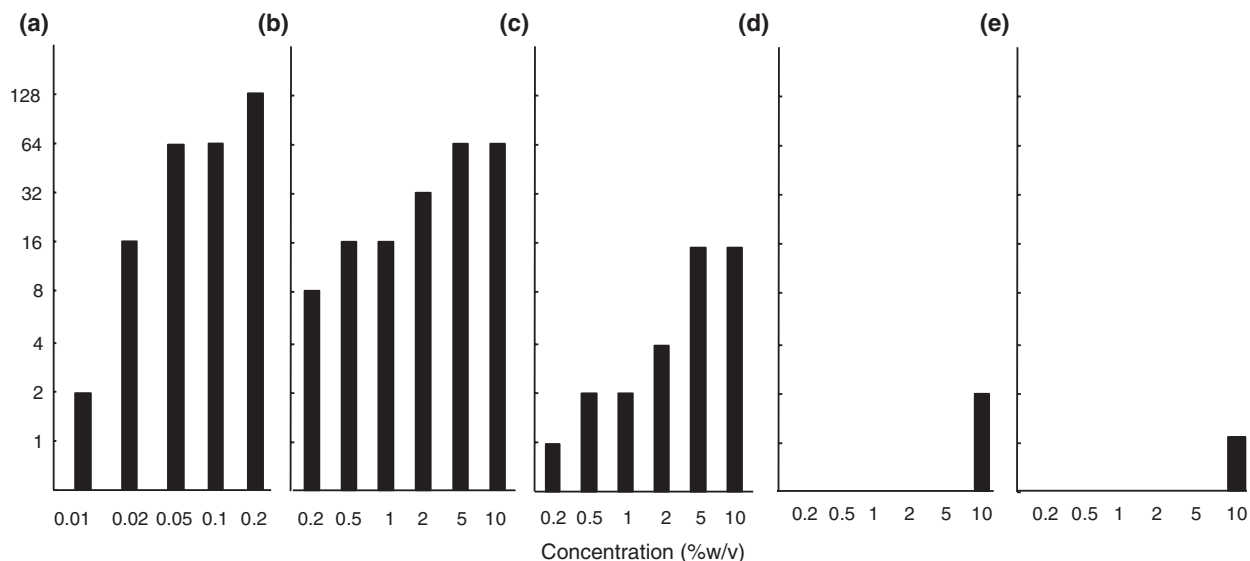


Fig. 2. IgE antibody responses following systemic exposure to peanut agglutinin, ovalbumin (OVA), bovine serum albumin (BSA), potato agglutinin or potato protein extract (PPE). Groups of mice ($n = 5$) received 0.25 mL of various concentrations of peanut agglutinin (a) (0.01–0.2%) or of OVA (b), BSA (c), potato agglutinin (d) or PPE (e) (0.2–10%) by i.p. injection on days 0 and 7. Seven days later, the animals were exsanguinated, and serum samples were pooled per treatment group and tested for the presence of specific IgE antibody by homologous passive cutaneous anaphylaxis assay. Negative control serum samples (from naïve animals) were tested concurrently and were routinely negative (data not shown). Data are expressed as reciprocal IgE titre. The statistical significance of differences between pairs of proteins in IgE titres derived from pooled serum samples was compared over the complete dose response by the Sign test. Peanut agglutinin was excluded from these analyses due to the different dose range utilized. Statistically significant differences ($P < 0.05$) were recorded between OVA and potato agglutinin and OVA and PPE and between BSA and potato agglutinin and BSA and PPE. The differences between potato agglutinin and PPE were not significant.

peanut agglutinin, did not induce significant IgE production demonstrates that not all agglutinins (or plant lectins) will stimulate IgE. In addition, this protein has been demonstrated to be very stable to digestion in SGF [25, 26], a suggested parameter for the characterization of allergenic potential [2, 7]. The theoretical basis for resistance to proteolytic digestion and an association with allergenicity is that allergens are stable proteins that therefore persist in the gastrointestinal tract in an intact form, allowing sufficient opportunity to interact with the immune system and to stimulate immune and allergic responses. This relationship between digestibility and allergenic activity is clearly not absolute, with some stable proteins failing to stimulate IgE antibody responses despite inducing robust IgG antibody responses. It remains to be confirmed that oral (gavage) rather than i.p. exposure to a stable protein such as potato agglutinin induces a similar antibody profile (high-titre IgG but low/not detectable IgE). However, in our experience [16, 21], proteins that differ in their ability to cause food allergy and IgE production when administered via a parenteral (i.p.) route display similar characteristics when administered by gavage. Thus, exposure of BALB/c strain mice to peanut agglutinin by gavage or i.p. administration in each case stimulated high-titre IgG and IgE antibody responses, whereas treatment with PPE failed to induce robust IgE antibody responses by either route of exposure [16]. Indeed, it appears that oral exposure to proteins may be somewhat less sensitive than i.p. administration with respect to the induction of robust IgE antibody responses. Thus, although in many experiments daily gavage exposure of BN rats to 1 mg/mL OVA resulted in 80% IgE responder animals, in a minority of experiments, the identical dosing schedule failed to induce specific IgE antibody in any recipient [14]. Some other

investigators have reported similar experiences when attempting to immunize rats via the oral route [16, 27]. Furthermore, in comparative experiments performed with BALB/c strain mice, it was observed that only weak and variable IgE antibody responses were elicited by OVA (a stable allergen) when given by gavage, whereas the same protein when administered by the i.p. route stimulated vigorous IgE antibody responses [16]. Given that parenteral exposure to protein apparently provides for greater sensitivity of detection of IgE responses, it is likely therefore that potato agglutinin would also fail to provoke detectable IgE if given by the oral route.

In these experiments, test proteins have been administered by i.p. injection, under which conditions the opportunities for the generation of vigorous immune responses are optimized. It may appear that in this context of food allergy, oral administration is the most appropriate route of exposure, given that it is the most likely route of sensitization for humans. However, as described above, gavage exposure of rats or mice to proteins can be variable or insensitive with respect to the induction of IgE antibody responses [14, 16]. Our approach has been to conduct these experiments in the absence of adjuvant, allowing assessment of the inherent potential of a given protein to induce allergic sensitization (IgE antibody responses). Other investigators have chosen to incorporate adjuvants, such as carrageenan or cholera toxin, which are reportedly selective for IgE antibody responses, into immunization protocols [11, 12, 28, 29]. While co-administration of adjuvant will undoubtedly increase the sensitivity of detection of IgE antibody responses to proteins, there is some concern that this may be at the cost of some loss in selectivity. There is limited evidence to suggest that adjuvant may override the inherent differences between

proteins with respect to their ability to induce IgE antibody, conferring on non-allergens the appearance of sensitizing potential [30]. An additional point of concern, and one that was highlighted in a recent report on the safety assessment of genetically modified plants by the Royal Society [31], is that a potential route of sensitization to novel allergens from gene products introduced into plants may be through exposure via the inhalation route to pollen. It may therefore be advantageous to demonstrate that any approach for the hazard characterization of potential allergens is able to identify proteins where the primary route of sensitization may be via inhalation. We have demonstrated previously that parenteral (i.p.) treatment with certain respiratory sensitizing detergent enzymes, including a lipase from *Aspergillus oryzae* and an amylase from *Bacillus subtilis*, elicited the production of relatively high-titre specific IgE antibody, suggesting that those proteins that have respiratory sensitizing capacity may also be characterized by their ability to stimulate IgE responses following systemic treatment of BALB/c strain mice [17].

It is assumed that protein allergens have certain properties that distinguish them from proteins that fail to cause allergic sensitization [32, 33]. Although it could be argued that all proteins are potential allergens, given appropriate levels and conditions of exposure, in practice this appears not to be the case as only a small proportion of the food proteins that individuals consume regularly is associated with allergic disease. Thus, in the USA and in Western Europe, most cases of food allergy are associated with a relatively limited range of produce, the most commonly implicated sources being peanuts, tree nuts, hen's egg, cow's milk, wheat, soybeans, fish and shellfish [34–37]. Furthermore, some protein allergens, such as those associated with allergy to peanuts, often cause persistent and severe sensitization reactions, whereas others (including, for instance, some milk proteins) are characterized by less profound and more transient sensitization [38–40]. Those proteins that have been identified as human allergens from foods such as the peanut that induce particularly severe and persistent reactions, are likely to be potent allergens and these may provide useful positive controls for the development of tests for allergenicity. Indeed, the recent recommendations of the FAO/WHO Expert Consultative Committee on Allergenicity of Foods Derived from Biotechnology [8] were that the allergenicity of novel proteins should be assessed against well-known strong and weak food allergens and non-allergenic proteins. Taken together, these observations suggest that there is indeed a spectrum of allergenicity among food proteins. However, it is difficult given the current knowledge base to assess the relative potency of individual proteins within foods and between foods with any degree of certainty. At present, the view is that although proteins cannot be ranked for allergenic potency with any degree of accuracy, some proteins are clearly allergenic, whereas other proteins, despite exposure through the diet, apparently lack allergenicity. Another related issue is that of inter-individual susceptibility to the development of sensitization. In common with other forms of atopic disease, the pathophysiology of food allergy is complex and inter-individual differences in susceptibility will be governed by a combination of environmental factors, genetic predisposition and exposure conditions,

including the age at which first exposure occurs [3]. Given this complexity, it is generally considered that it will not prove possible to define a method using experimental animals that will accurately predict the likely prevalence, persistence and severity of food allergy among human populations exposed to a novel allergen in the diet. While this is undoubtedly true, the purpose of the approach described herein, and indeed the first step of any safety assessment process, is to identify intrinsic hazard, or the lack of it. In this case, the intrinsic hazard is identified as a function of the ability of the protein to induce IgE antibody production, and the next steps in the risk assessment process will be to characterize the nature of the hazard and to define potential risks to humans based on likely exposure patterns.

In conclusion, the data presented here suggest that the measurement of antibody (IgE) responses in BALB/c mice appears to identify allergens accurately and to distinguish them from those materials that apparently lack allergenicity. Marked and very significant differences in the ability of allergens to stimulate IgE responses have been demonstrated over a wide dose range under conditions where non-allergens are clearly of equivalent immunogenicity with respect to the induction of IgG antibody. Further investigations with a wider range of proteins are required, but experience to date suggests that this approach could play an important role in the identification and characterization of the allergenic potential of novel proteins.

References

- 1 Taylor SL, Hefle SL. Will genetically modified foods be allergenic? *J Allergy Clin Immunol* 2001; 107:765–71.
- 2 Metcalfe DD, Astwood JD, Townsend R, Sampson HA, Taylor SL, Fuchs RL. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Crit Rev Food Sci Nutr* 1996; 36 (Suppl.):S165–86.
- 3 Kimber I, Dearman RJ. Food allergy: what are the issues? *Toxicol Lett* 2001; 120:165–70.
- 4 Lack G, Chapman M, Kalsheker N, King V, Robinson C, Venables K. Report on the potential allergenicity of genetically modified organisms and their products. *Clin Exp Allergy* 2002; 32:1131–43.
- 5 Anderson JA. Allergic reactions to foods. *Crit Rev Food Sci Nutr* 1996; 36 (Suppl.):S19–38.
- 6 Hourihane JO'B. Prevalence and severity of food allergy – need for control. *Allergy* 1998; 53 (Suppl.):84–8.
- 7 Astwood JD, Leach JN, Fuchs RL. Stability of food allergens to digestion *in vitro*. *Nat Biotechnol* 1996; 14:1269–73.
- 8 Food and Agriculture Organization/World Health Organization (FAO/WHO). Report of a joint FAO/WHO Expert Committee on Allergenicity of Foods derived from Biotechnology. Rome, Italy FAO/WHO January 22–25, 2001
- 9 Ermel RW, Kock M, Griffey SM, Reinhart GA, Frick OL. The atopic dog: a model for food allergy. *Lab Anim Sci* 1997; 47:40–9.
- 10 Helm RM, Furuta GT, Stanley JS et al. A neonatal swine model for peanut allergy. *J Allergy Clin Immunol* 2002; 109:136–42.
- 11 Atkinson HAC, Johnson IT, Gee JM, Grigoriadou F, Miller K. Brown Norway model of food allergy: effect of plant components on the development of oral sensitization. *Food Chem Toxicol* 1996; 34:27–32.
- 12 Atkinson HAC, Miller K. Assessment of the Brown Norway rat as a suitable model for the investigation of food allergy. *Toxicology* 1994; 91:281–8.

- 13 Knippels LMJ, Penninks AH, van Meeteren M, Houben GF. Humoral and cellular responses in different rat strains on oral exposure to ovalbumin. *Food Chem Toxicol* 1999; 37:881–8.
- 14 Knippels LMJ, Penninks AH, Spanhaak S, Houben GF. Oral sensitization to food proteins: a Brown Norway rat model. *Clin Exp Allergy* 1998; 28:368–75.
- 15 Dearman RJ, Caddick H, Basketter DA, Kimber I. Divergent antibody isotype responses induced in mice by systemic exposure to proteins: a comparison of ovalbumin with bovine serum albumin. *Food Chem Toxicol* 2000; 38:351–60.
- 16 Dearman RJ, Caddick H, Stone S, Basketter DA, Kimber I. Characterization of antibody responses induced in rodents by exposure to food proteins: influence of route of exposure. *Toxicology* 2001; 167:217–31.
- 17 Hilton J, Dearman RJ, Basketter DA, Kimber I. Serological responses induced in mice by immunogenic proteins and protein respiratory allergens. *Toxicol Lett* 1994; 73:43–53.
- 18 Metcalfe DD. Food allergens. *Crit Rev Allergy* 1985; 3:331–9.
- 19 Burks AW, Cockrell G, Connaughton C, Guin J, Allen W, Helm RM. Identification of peanut agglutinin and soybean trypsin inhibitor as minor legume allergens. *Int Arch Allergy Immunol* 1994; 105:143–9.
- 20 Jeannot-Peter N, Piletta-Zanin PA, Hauser C. Facial dermatitis, contact urticaria, rhinoconjunctivitis, and asthma induced by potato. *Am J Contact Dermatol* 1999; 10:40–2.
- 21 Dearman RJ, Caddick H, Stone S, Kenna JG, Basketter DA, Kimber I. Immunogenic properties of rapidly digested food proteins following gavage exposure of mice: a comparison of ovalbumin with a potato acid phosphatase preparation. *Food Chem Toxicol* 2002; 40:625–33.
- 22 Statistical Analysis System (SAS) Institute Inc., SAS/SAT User's Guide, Version 8. Cary, NC: SAS Institute Inc. 1999
- 23 Fiocchi A, Restani P, Riva E et al. Meat allergy. I. Specific IgE to BSA and OSA in atopic beef sensitive children. *J Am College Nutr* 1995; 14:239–44.
- 24 Joliat TL, Weber RW. Occupational asthma and rhinoconjunctivitis from inhalation of crystalline bovine serum albumin powder. *Ann Allergy* 1991; 66:301–4.
- 25 Atherton KT, Dearman RJ, Kimber I. Protein allergenicity in mice: a potential approach for hazard identification. *Ann NY Acad Sci* 2002; 964:163–72.
- 26 Dearman RJ, Kimber I. Determination of protein allergenicity: studies in mice. *Toxicol Lett* 2001; 120:181–6.
- 27 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.
- 28 Li X-M, Schofield BH, Huang C-K, Kleiner GI, Sampson HA. A murine model of IgE-mediated cow's milk hypersensitivity. *J Allergy Clin Immunol* 1999; 103:206–14.
- 29 Li X-M, Serebrisky D, Lee S-Y et al. A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. *J Allergy Clin Immunol* 2000; 106:150–8.
- 30 Dearman RJ, Basketter DA, Kimber I. Anti-protein antibody isotype distribution: influence of adjuvant. *Immunol Lett* 1999; 69:102 [Abstract].
- 31 The Royal Society., Genetically Modified Plants for Food Use and Human Health – An Update, 2002.
- 32 Huby RDJ, Dearman RJ, Kimber I. Why are some proteins allergens? *Toxicol Sci* 2000; 55:84–8.
- 33 Taylor SL, Lehrer SB. Principles and characteristics of food allergens. *Crit Rev Food Sci Nutr* 1996; 36 (Suppl.):S91–118.
- 34 Bush RR, Hefle SL. Food allergens. *Crit Rev Food Sci Nutr* 1996; 36 (Suppl.):S119–63.
- 35 Hefle SL, Nordlee JA, Taylor SL. Allergenic foods. *Crit Rev Food Sci Nutr* 1996; 36 (Suppl.):S69–89.
- 36 Sampson HA. IgE-mediated food intolerance. *J Allergy Clin Immunol* 1988; 81:495–504.
- 37 Young E, Stoneham MD, Petrukevitch A, Barton J, Rona R. A population study of food intolerance. *Lancet* 1994; 343:1127–30.
- 38 Bock SA, Munoz-Furlong A, Sampson HA. Fatalities due to anaphylactic reactions to foods. *J Allergy Clin Immunol* 2001; 107:191–3.
- 39 Pumphrey RS. Lessons for management of anaphylaxis from a study of fatal reactions. *Clin Exp Allergy* 2000; 30:1144–50.
- 40 Spergel JM, Beausoleil JL, Pawlowski NA. Resolution of childhood peanut allergy. *Ann Allergy Asthma Immunol* 2000; 85:473–6.