

ORIGINAL PAPER

Influence of plant lipids on immune responses in mice to the major Brazil nut allergen Ber e 1

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Clinical and
Experimental
Allergy

Summary

Background Lipids, particularly bacterial lipopolysaccharide, can impact on immune responses to proteins, with low doses enhancing type 2 responses.

Objective We have examined the influence of natural plant lipid extracts on antibody responses provoked in mice by recombinant Ber e 1, the major allergen in Brazil nuts.

Methods BALB/c strain mice were immunized (by intraperitoneal injection) with natural or recombinant Ber e 1 produced in *Pichia pastoris* and admixed with various lipid fractions isolated from Brazil nuts. Serum samples were analysed for specific IgE antibody by homologous passive cutaneous anaphylaxis assay and for IgG by enzyme-linked immunosorbant assay.

Results Exposure to recombinant (lipid-free) Ber e 1 alone failed to induce detectable IgG or IgE antibody. Co-administration of the total lipid fraction (with reduced triglyceride levels), sterol-rich, or polar lipid fractions, resulted in marked adjuvant effects on IgG and IgE. However, the β -sitosterol and glycolipid-rich fractions were associated with only low-level IgG antibody, and had little impact on IgE antibody production. Natural Ber e 1 containing endogenous lipids also provoked IgG and IgE antibody responses. Identical IgE and IgG antibody responses were detected regardless of whether natural or recombinant Ber e 1 was used as substrates for analyses.

Conclusion Endogenous Brazil nut lipids are required for the induction of optimal antibody responses to Ber e 1 in the BALB/c strain mouse. Appropriate antibody binding sites are present on both natural and recombinant forms of Ber e 1, suggesting that the impact of lipid is at the induction phase, rather than antibody recognition, and is possibly required for efficient antigen presentation.

Keywords Ber e 1, IgE, IgG, plant lipids, protein allergenicity

Submitted 26 April 2006; revised 30 October 2006; accepted 29 December 2006

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Introduction

Food allergy is an important health issue, the prevalence of which has been estimated recently in the USA at 3.5–4% of adults, and approximately 6–8% of young children and infants [1, 2]. Although cell-mediated immune reactions play a major role in some forms of food intolerance (e.g. in coeliac disease associated with gluten sensitivity) [3], it is IgE antibody that is the key biological mediator of food allergic reactions [2, 4]. It has been recognized for some time now that the initiation and maintenance of IgE antibody production are dependent

upon the development of a selective type 2 immune response. The qualitative characteristics of adaptive immune responses, including allergic responses, are governed largely by functional heterogeneity among T lymphocytes, particularly those of the CD4⁺ T helper (Th) cell subset [5, 6]. The most differentiated subsets of Th cells, designated Th1 and Th2, develop during the evolution of immune responses and display phenotypes of selective cytokine secretion. Th2 cells produce cytokines (including IL-4, IL-5, IL-10 and IL-13) that are required for the development of IgE antibody responses, and that facilitate the elicitation of IgE-mediated allergic reactions [7, 8].

The development of allergic sensitization to food proteins will be favoured therefore by conditions that permit the selective generation of Th2-type immune responses and IgE antibody production. Among the factors that impact on the acquisition of allergy, and, it is assumed, the preferential activation of Th2-type cells, are properties that are intrinsic to the protein itself. In theory, it can be argued that all proteins are potentially allergenic if the levels of exposure are high enough, and the conditions of exposure appropriate. In practice, however, this appears not to be the case. Relatively few foods and specific protein families are commonly implicated as food allergens, despite the huge variety of food products encountered in normal diets [9–11]. Furthermore, protein allergens appear to differ with respect to the nature of the sensitization they elicit. For example, peanut allergens often cause persistent and severe allergic reactions, whereas others (including for instance some cows' milk proteins) are associated with more transient sensitization and less marked reactions [12–14]. These observations suggest that there is a spectrum of allergenic properties among proteins, with many failing to cause the acquisition of allergic sensitization, despite being inherently immunogenic. A minority of proteins is both immunogenic and can provoke the quality of immune response necessary for the acquisition of allergic sensitization.

There is considerable interest in defining the characteristics that confer sensitizing potential on such allergenic proteins [15–17]. Several features are thought to be important in this respect. Included among these are: size and number of IgE-binding sites, resistance to proteolytic digestion and heat stability, glycosylation status, biological function (enzymatic activity, storage protein, etc.), overall immunogenicity, and the ways in which the protein is recognized, internalized and processed for presentation to the immune system. For example, most allergens have more than two IgE-binding sites; the peanut allergen Ara h 1 and the major soybean allergen, Gly m Bd, having 23 and six linear epitopes, respectively [18, 19]. Many of the important allergens are also heat stable, retaining their allergenicity after heating [20], and most are relatively resistant to proteolysis by simulated gastric fluid (SGF) compared with control (non-allergenic) proteins [21]. However, none of these properties is unique to allergens [15, 20].

In addition to consideration of the intrinsic properties of potential protein allergens, there is increasing interest in the effects of extrinsic factors present within the food matrix that may impact on the development of allergic responses. It has been reported recently that the presence of bacterial lipopolysaccharide (LPS), a common contaminant of foods, can impact significantly on the quality of immune response provoked by protein allergens [22]. Thus, intra-nasal exposure of mice to the chicken egg allergen, ovalbumin (OVA), in the presence of low levels of

Escherichia coli LPS (0.1 µg/mouse) was found to enhance type 2 immune responses, whereas high-dose endotoxin (100 µg/mouse) stimulated type 1 responses. Low-dose endotoxin provoked vigorous IgE and IgG1 serum antibody production, type 2 cytokine (IL-4, IL-5 and IL-13) expression by draining lymph node cells, and challenge-induced airways inflammation with a predominant eosinophil infiltrate. In contrast, high dose LPS stimulated high titre IgG2a antibody responses, IFN-γ secretion and challenge-induced airways inflammation with marked neutrophil infiltration; hallmarks of a type 1 immune response. Other investigators have reported that immunization with the whole food extract enhanced the immunogenicity of peanut allergens compared with exposure to the purified protein alone, although the nature of the component or components responsible for this adjuvant effect was not resolved [23].

In previous studies, we have demonstrated that following intraperitoneal (ip) administration, and in the absence of adjuvant, proteins display marked differences in their intrinsic capacity to induce IgE antibody responses that are largely consistent with what is known of their relative sensitizing activity [24]. Here, we have used this experimental approach to explore the impact of natural plant lipids derived from the Brazil nut on the sensitizing potential of the major Brazil nut allergen Ber e 1. To this end, antibody responses provoked by natural Ber e 1 have been compared with those elicited by either lipid-free recombinant Ber e 1 alone, or recombinant Ber e 1 admixed with various Brazil nut lipid fractions. It has been reported that the lipid fraction represents a major constituent of the edible portion of the Brazil nut, with the average composition being approximately 14% protein and 66% lipids [25, 26]. Interestingly, the fatty acid composition of the lipid fraction has also been characterized as approximately 25% saturated fatty acids, 29% monounsaturated fatty acids and 45% polyunsaturated fatty acids [25, 26] with the latter having been implicated previously as potential immunomodulators.

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 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) and bovine serum albumin (BSA; Grade V, 98% pure) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Natural Ber e 1 protein from Brazil nut seeds was prepared as described previously using a Sephadex 75 size exclusion column [27] (Pharmacia, 2.5 cm × 90 cm; Amersham, Little Chalfont, UK); such material was approximately 50% protein and 50% lipids (termed 'partially purified'). Correctly folded recombinant Ber e 1 was produced in *Pichia pastoris* and prepared as previously described [28]. In order to remove endogenous lipids, both natural and recombinant Ber e 1 proteins were further purified by high-performance liquid chromatography (HPLC) using a preparative stainless-steel C8 reverse phase column [29] (termed 'highly purified' or 'lipid-free'). The reverse-phase purified protein samples were freeze dried and stored at -20 °C until further analysis. Total lipids from Brazil nut seeds were extracted using chloroform : methanol according to standard procedures [30]. The various lipid classes were produced by preparative thin-layer chromatography [31] and quantified gravimetrically (by weight) following solvent evaporation. The estimated composition of the lipid fractions were as follows: (i) intermediary lipids enriched mainly with steryl glucosides and digalactosyl-diglycerides, designated 'glycolipid-rich'; (ii) Brazil nut β -sitosterol; (iii) total lipid fraction with reduced triglyceride levels; (iv) mainly intermediary polarity lipids (sterols, sterol ester, free fatty acids), designated 'sterol-rich'; (v) mainly polar lipids (PE, PC, PI and sulpholipids). The total amount of each lipid fraction was measured gravimetrically and calculated using the molecular mass of dioleoyl phosphatidyl choline as standard average mass. Reconstituted protein-lipid fractions were prepared by mixing 8 mL of 1.6% (w/v) recombinant Ber e 1 in water with 1 mL of lipid fraction dissolved in methanol using a lipid-protein ratio of 1:1 with respect to the mass calculated using dioleoyl phosphatidyl choline as standard average reference mass. The reconstituted lipid-Ber e 1 suspensions were then frozen, freeze dried and maintained at -20 °C until further use.

Sensitization procedure

Groups of mice ($n = 5$) received 250 μ L of 20 or 10 mg/mL of natural or recombinant Ber e 1 or OVA in phosphate-buffered saline (PBS) by ip injection on days 0 and 7. In some experiments, recombinant Ber e 1 was admixed with various lipid fractions prepared from Brazil nuts as described above. 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 immunoglobulin G, immunoglobulin G1 and immunoglobulin G2a antibody analyses

Protein-specific IgG, IgG1 and IgG2a antibodies were detected using ELISA. Plastic microtitre plates (Nunc, Copenhagen, Denmark) were coated with 100 μ 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. Doubling dilutions of mouse serum samples 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 sheep anti-mouse IgG (Serotec, Kidlington, UK) diluted 1:4000 with 1% BSA in PBS, or with peroxidase-labelled rat monoclonal anti-mouse IgG1 or anti-mouse IgG2a antibody (Serotec) 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 (OD) 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 > 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 (log₂) for each treatment group.

Measurement of anti-protein IgE antibody

The presence in mouse serum of IgE antibodies was detected by 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 μ L) 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 μ L 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 μ L of serum is required in order to determine the IgE titre. Owing to 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 PBS were injected (30 μ L) 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 PBS 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 PBS were injected intravenously. Thirty minutes 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 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 IgE antibody if the mean diameter of the challenge-induced cutaneous reaction was >3 mm.

Results

In initial experiments, the ability of systemic exposure to natural Ber e 1 to induce antibody responses in BALB/c strain mice was investigated. Mice ($n=4$ /group) were immunized by ip injection with 250 μ L of 20 mg/mL of either partially purified (50% protein), or endogenous lipid free highly purified natural Ber e 1 extract. Serum samples were analysed for IgE content by homologous PCA assay (Fig. 1). The number of IgE responders per group was determined by analysis of undiluted individual serum samples using the partially purified natural Ber e 1 preparation as substrate. In each of two independent

experiments, two of four mice were IgE responders following exposure to partially purified natural Ber e 1. Exposure to highly purified natural Ber e 1 that had been completely depleted of endogenous lipid failed to stimulate detectable IgE antibody production (0/4 responders) (Fig. 1a). Titres of IgE antibody were determined on an experimental group basis by PCA analyses of serial doubling dilutions of pooled serum samples (Fig. 1b). Consistent with the individual IgE responder data, highly purified Ber e 1 failed to stimulate a positive PCA reaction when tested neat (thus, resulting in a titre of <1). In both independent experiments, exposure to partially purified Ber e 1 provoked positive PCA reactions in serum diluted 1:2, 1:4 and 1:8 (Fig. 1b). There was some inter-experimental variation in the vigour of IgE responses, with a titre of 1:8 recorded in one experiment, whereas in the second experiment serum also tested PCA-positive at dilutions of 1:16 and 1:32 (resulting in a recorded titre of 1:32). In repeat analyses using the endogenous lipid free highly purified preparation, an identical pattern of IgE antibody responses was recorded, with serum samples from animals immunized with the partially purified material provoking positive responses in the PCA assay whereas immunization with the lipid-free protein failed to stimulate detectable IgE.

The IgG antibody content of the serum samples was analysed by ELISA (Fig. 2; individual serum samples). Treatment with lipid-free highly purified natural Ber e 1 failed to induce detectable IgG antibody in any animal

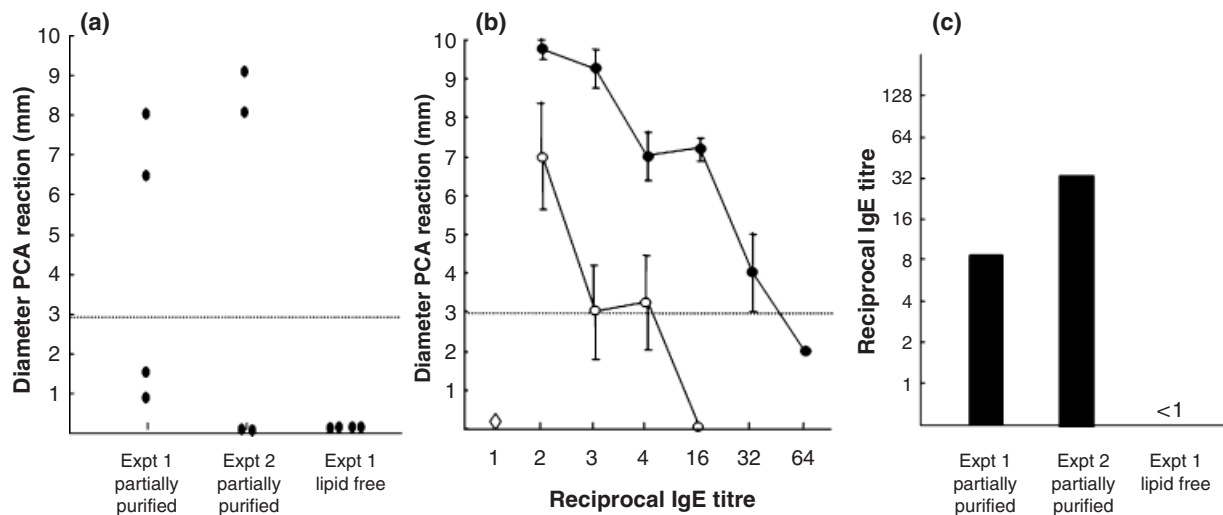


Fig. 1. Influence of endogenous plant lipids on IgE antibody responses induced by intraperitoneal exposure to natural Ber e 1. Animals ($n=4$) received 250 μ L of 20 mg/mL of partially purified natural Ber e 1 extract (50% protein; experiments 1 and 2) or endogenous lipid free highly purified natural Ber e 1 extract (experiment 1) by intraperitoneal injection on days 0 and 7. Seven days later animals were exsanguinated, and individual (a) and pooled (b) serum samples tested for the presence of specific IgE antibody by homologous passive cutaneous anaphylaxis (PCA) assay using the partially purified material as substrate. Negative control serum samples (from naïve animals) were tested concurrently and were routinely negative (data not shown). Individual serum samples were tested neat to determine number of IgE responder animals; data are displayed as mean diameter of PCA reaction for each serum sample (a). Pooled serum samples were serially diluted to determine IgE titre on a treatment group basis. Data are displayed as mean and SE of PCA reaction ($n=4$) for each dilution tested (b; ○ = partially purified, experiment 1; ● = partially purified, experiment 2; ◇ = highly purified, experiment 1) (b). Horizontal line denotes the cut-off for a positive PCA reaction; 3 mm.

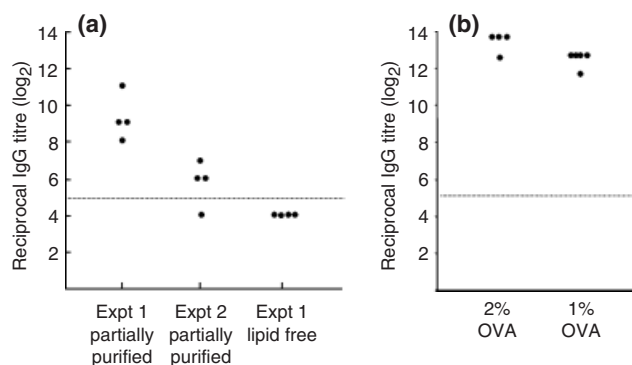


Fig. 2. Influence of endogenous plant lipids on IgG antibody responses induced by intraperitoneal exposure to natural Ber e 1. Animals ($n = 4$) received 250 μ L of 20 mg/mL of partially purified natural Ber e 1 (50% protein; experiments 1 and 2) or endogenous lipid free highly purified natural Ber e 1 extract (experiment 1) by intraperitoneal injection on days 0 and 7 (a). Control animals received 250 μ L of 20 or 10 mg/mL of ovalbumin (OVA) by intraperitoneal injection on days 0 and 7 (b). Seven days later animals were exsanguinated, and individual serum samples 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 IgG reciprocal titre (log₂) for each individual serum sample. Horizontal line denotes the highest serum concentration tested [1 in 16; 5 (log₂)].

(titre < 1 : 16 in each case). Exposure to the partially purified natural Ber e 1 resulted in IgG antibody responses in each of the two independent experiments, with titres ranging from 1 : 32 to 1 : 1024. There was some inter-experimental variation in IgG antibody levels, with more vigorous responses recorded in experiment 1 than in experiment 2, the latter of which included one serum sample in which IgG was not detected at the maximum concentration tested (1 : 16). Comparisons with antibody responses induced by an equivalent dose of the common human food allergen OVA (20 mg/mL) revealed that IgG antibody responses to this protein are considerably more vigorous, with maximal titres of 1 : 8192 achieved (Fig. 2b). Interestingly, however, despite the relatively low immunogenicity of natural Ber e 1 compared with OVA, similar IgE antibody titres have been achieved previously, with the dose of OVA used in the present investigations generally provoking IgE titres of 1 : 32 [24].

Given the apparent adjuvant effect of endogenous plant lipids from Brazil nuts, the following lipid fractions were isolated according to their polarity on a silica gel system. Following this isolation procedure most of the fractions with one exception are mixtures of complex lipids. Owing to the extensive literature on immunomodulatory effects of plant sterols, β -sitosterol was purified in sufficient amounts to be considered an isolated class. These were: (i) 'glycolipid-rich' lipids (steryl glucosides and digalactosyldiglycerides); (ii) Brazil nut β -sitosterol; (iii) total lipid fraction with reduced triglyceride levels; (iv) intermediary polarity lipids (sterols, sterol ester, free fatty acids), designated 'sterol-rich'; and (v) polar lipids. Recombinant

Ber e 1 (expressed in *P. pastoris*), purified by reverse phase chromatography, was admixed with the various lipid fractions in a 1 : 1 molar stoichiometry with respect to the amount of lipid calculated according to the theoretical molecular mass of dioleoyl phosphatidyl choline. Mice (BALB/c strain; $n = 5$ /group) were exposed ip to 20 mg/mL of recombinant Ber e 1 in the presence or absence of endogenous plant lipid fractions. Undiluted individual serum samples were analysed for IgE antibody responses using natural Ber e 1 as substrate. The number of responders per group was recorded (Fig. 3a). Treatment with recombinant Ber e 1 alone, or supplemented with the glycolipid-rich fraction failed to stimulate IgE antibody production (zero of five IgE responders). Recombinant Ber e 1 in the presence of the β -sitosterol lipid fraction (ii) induced IgE antibody responses in one of five animals. In contrast, the presence of total lipids (containing reduced triglyceride levels), sterol-rich and polar lipid fractions [fractions (iii), (iv) and (v)], each provoked detectable IgE antibody responses in three of five animals. In subsequent analyses, undiluted serum samples pooled on an experimental group basis were analysed for IgE content by PCA, using as substrate either recombinant or natural (highly purified) Ber e 1 (Fig. 3b). Serum derived from animals treated with recombinant Ber e 1 alone, or in the presence of glycolipid-rich or β -sitosterol [fractions (i) and (ii)]; lacked detectable IgE antibody. Positive IgE responses were recorded with mice exposed to recombinant Ber e 1 supplemented with total lipids, sterol-rich and polar lipid fractions [fractions (iii), (iv) and (v)]. Identical results were achieved regardless of whether recombinant or natural Ber e 1 was used as substrate. In these samples, titres of 1 were achieved (samples diluted 1 : 2 and 1 : 4 were negative in the PCA assay; data not shown).

Levels of IgG antibody in individual serum samples were analysed by ELISA (Fig. 4). Exposure to recombinant Ber e 1 alone or in the presence of the glycolipid-rich fraction failed to stimulate detectable IgG antibody in the majority of animals. Ber e 1 supplemented with β -sitosterol, total lipids (containing reduced triglycerides), sterol-rich and polar lipid fractions [fractions (ii), (iii), (iv) and (v)] stimulated IgG antibody production in all animals, although antibody titres showed some inter-animal variation, ranging from 1 : 16 to 1 : 2048. Finally, the subclass distribution of the IgG antibody response (with respect to IgG1 and IgG2a) was investigated (Fig. 5) using serum samples pooled on a group basis. In general IgG1 antibody responses paralleled total IgG antibody expression. Recombinant Ber e 1 did not induce detectable IgG1 antibody production. There was some adjuvant effect of the glycolipid-rich fraction with respect to IgG1 titre, whereas the other lipid fractions each induced more vigorous IgG1 antibody responses compared with those provoked by partially purified natural Ber e 1 (containing endogenous lipids). Interestingly, immunization with natural Ber e 1

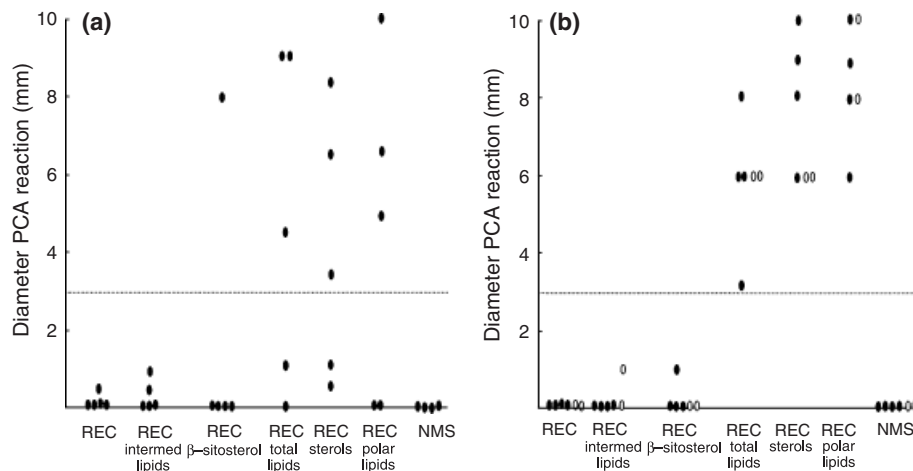


Fig. 3. Influence of endogenous plant lipids on IgE antibody responses induced by intraperitoneal exposure to recombinant Ber e 1. Animals ($n = 5$) received 250 μ L of 20 mg/mL of recombinant Ber e 1 alone or supplemented with various lipid fractions were isolated from Brazil nuts [(i) 'glycolipid-rich'; (ii) Brazil nut β -sitosterol; (iii) total lipid fraction with reduced triglyceride levels; (iv) intermediary polarity lipids (sterols, sterol ester, free fatty acids), designated 'sterol-rich'; (v) polar lipids] in a 1 : 1 stoichiometry (with respect to the mass calculated using dioleoyl phosphatidyl choline as standard average reference mass) by intraperitoneal injection on days 0 and 7. Seven days later animals were exsanguinated, and individual serum samples tested for the presence of specific IgE antibody by homologous passive cutaneous anaphylaxis (PCA) assay. (a) Individual serum samples were tested neat to determine number of IgE responder animals using recombinant Ber e 1 as substrate; data are displayed as mean diameter of PCA reaction for each serum sample (tested in $n = 2$ animals). (b) Pooled serum samples were analysed (undiluted) for IgE content in four animals (using recombinant Ber e 1 substrate; ●) or in two animals (using natural Ber e 1 substrate; ○); data are displayed as diameter of PCA reaction in each individual animal tested (b). Negative control serum samples (from naïve animals; naïve mouse serum, NMS) were tested concurrently. Horizontal line denotes the cut-off for a positive PCA reaction; 3 mm.

also stimulated IgG2a antibody production: IgG2a being an antibody isotype that in mice is associated with Th1-type cell activation. However, the IgG2a antibody titre was 1 : 128, considerably lower than the corresponding IgG1 antibody titre (1 : 4096) and resulting in a ratio of IgG1 to IgG2a antibody of 32 : 1. IgG2a antibody production was also induced following administration of recombinant Ber e 1 supplemented with total lipids (containing reduced triglyceride levels), sterol-rich and polar lipid fractions [fractions (iii), (iv) and (v)]; IgG1 to IgG2a antibody ratios were similar to that provoked by the native protein containing endogenous lipids (16 : 1 to 64 : 1). In contrast, exposure to recombinant Ber e 1 in the presence of the glycolipid-rich or β -sitosterol [fractions (i) and (ii)] provoked a more type 2 IgG subclass profile, with relatively little IgG2a antibody, resulting in a ratio of IgG1 to IgG2a of 512 : 1.

Discussion

Currently, there is considerable interest in definition of the factors that confer on food proteins the ability to induce allergic sensitization [10, 15, 32, 33]. This has assumed increased importance with the need to identify and characterize potential food allergens as an essential part of the food safety assessment process, particularly as it relates to foods derived from genetically modified crop plants [34–36]. Included among the factors that appear to impact on the sensitizing properties of proteins are

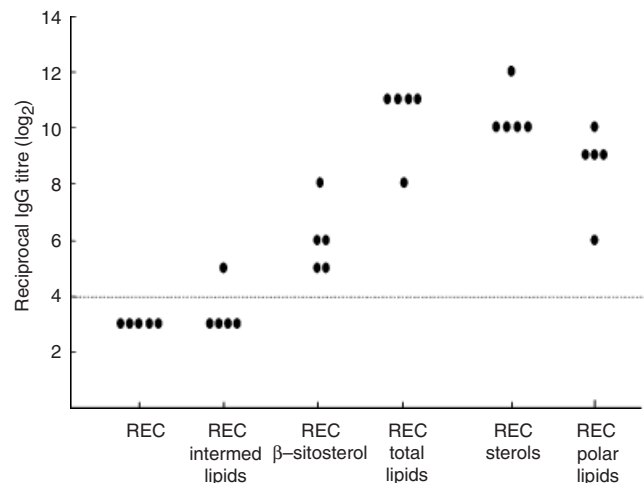


Fig. 4. Influence of endogenous plant lipids on IgG antibody responses induced by intraperitoneal exposure to recombinant Ber e 1. Animals ($n = 5$) received 250 μ L of 20 mg/mL of recombinant Ber e 1 alone or supplemented with various lipid fractions were isolated from natural Brazil nuts [(i) 'glycolipid-rich'; (ii) Brazil nut β -sitosterol; (iii) total lipid fraction with reduced triglyceride levels; (iv) intermediary polarity lipids (sterols, sterol ester, free fatty acids), designated 'sterol-rich'; (v) polar lipids] in a 1 : 1 stoichiometry (with respect to the mass calculated using dioleoyl phosphatidyl choline as standard average reference mass) by intraperitoneal injection on days 0 and 7. Seven days later animals were exsanguinated, and individual serum samples 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 IgG reciprocal titre (log2) for each individual serum sample. Horizontal line denotes the highest serum concentration tested [1 in 8; 4 (log2)].

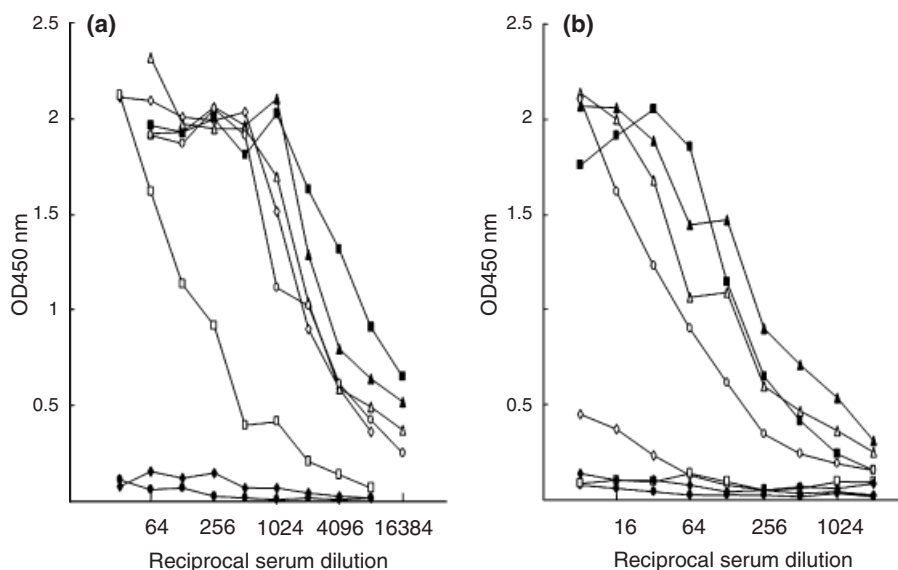


Fig. 5. Influence of endogenous plant lipids on IgG subclass antibody responses induced by intraperitoneal exposure to recombinant Ber e 1. Animals ($n = 5$) received 250 μ L of 20 mg/mL of recombinant Ber e 1 alone (●) or supplemented with various lipid fractions were isolated from native Brazil nuts [(i) 'glycolipid-rich' (□); (ii) Brazil nut β -sitosterol (◇); (iii) total lipid fraction with reduced triglyceride levels (■); (iv) intermediary polarity lipids (sterols, sterol ester, free fatty acids), designated 'sterol-rich' (▲); (v) polar lipids (△)] in a 1 : 1 stoichiometry (with respect to the mass calculated using dioleoyl phosphatidyl choline as standard average reference mass) by intraperitoneal injection on days 0 and 7. Seven days later animals were exsanguinated, and pooled serum samples tested for the presence of specific IgG1 (a) and IgG2a (b) antibody by ELISA. Negative control serum samples (from naïve animals) (◆) and positive control serum from animals exposed to 2% natural Ber e 1 (○) were tested concurrently. Data are displayed as optical density (OD) 450 nm vs. reciprocal serum dilution.

molecular size, resistance to proteolytic digestion, glycosylation status, functional properties and the way in which protein is processed and presented to the immune system [31, 33]. However, there is interest also in the possibility that factors extrinsic to the protein *per se* may also influence the acquisition of sensitization, and in this context one particular focus of attention has been the macromolecular matrix within which food proteins are encountered [37]. The data presented here lend further support the view that the food matrix has a potentially important impact on sensitization, and that with respect to Brazil nut allergy lipids may play a decisive role.

Despite the fact that the production of recombinant disulphide-rich proteins such as Ber e 1 in the correct conformation is technically demanding we have shown previously that properly folded protein which is immunogenically similar to the natural proteins can be obtained using eukaryotic expression [28, 38]. Consistent with previous reports [29] in the current series of experiments the material has been prepared successfully such that IgG and IgE-binding epitopes have remained intact and equivalent to the natural protein. An observation of particular interest deriving from these investigations was the failure of lipid-free Ber e 1 (either natural or recombinant) to elicit in BALB/c mice an IgE antibody response, or even a detectable IgG response under the conditions of analysis employed here. These data are in contrast to previous experience with known food allergens that,

when administered to mice of this strain under identical conditions of exposure, were found to provoke both IgG and IgE antibody responses [24, 39]. The results are unexpected, not least because Ber e 1 is well established as the major allergen of Brazil nuts [40], and this protein has the physicochemical attributes, including relative resistance to proteolytic digestion, frequently associated with food allergens [28, 29, 41].

The apparent inability of lipid-free Ber e 1 to provoke in mice immune responses consistent with sensitizing activity naturally raises questions about the utility of this approach for the prospective identification of potentially allergenic proteins. However, in practice this may not represent a problem. The currently favoured predictive mouse model [24, 39, 42, 43] is based upon assessment of test proteins (administered by ip injection) to elicit IgE antibody responses under conditions where there has been a clear induction of IgG antibody. The method therefore seeks to distinguish between immunogenicity and allergenicity. As such, proteins that fail to induce IgE antibody production under conditions where exposure has clearly resulted in an immunogenic response (as judged by serum IgG antibody) are judged to lack significant sensitizing activity. In the case of Ber e 1, the inability of lipid-free preparations of the protein to elicit either IgG or IgE antibody responses would signal concern that immunogenicity had not been achieved thereby avoiding an inappropriate conclusion being reached about the absence

of sensitizing potential. Recent independent investigations of responses in mice to peanut allergens have drawn similar conclusions regarding the importance of food matrices in the elicitation of IgE antibody responses [23].

The question remains, however, why in the absence of lipid Ber e 1 fails to engage the immune system, and the mechanisms through which this is facilitated by some lipids. It is now acknowledged that the ubiquitously expressed Toll-like receptors (TLR) play pivotal roles in host resistance to infectious disease via recognition of conserved molecular structures on potentially pathogenic micro-organisms. There is compelling evidence that ligand-activation of TLR, and particularly activation of TLR2/TLR4 by LPS, lipoproteins and fatty acids, can impact substantially on immune responses, including the elicitation of IgE antibody [22, 44–50]. Of particular interest are the observations that compared with BALB/c strain mice, the induction of type 2 cytokine responses and anaphylactic responses induced by intragastric administration of peanut allergen was considerably more vigorous in LPS-resistant (C3H/HeJ strain) mice, suggesting a role for TLR-4 in the severity of the allergic response [51, 52]. The available evidence indicates that the ligation of TLR may influence the vigour and quality of adaptive immune function through a variety of mechanisms, including modulation of dendritic cell [47] and mast cell [49] function, and via alterations in cytokine expression profiles [47, 50]. It is possible that any one or more of these immune parameters may have been impacted in these investigations by the natural plant lipids from Brazil nut that were found to enhance responses to Ber e 1. Definition of the relevant mechanisms will require further study.

However, another possible mechanism that should be considered is via the stimulation of specific immune responses to certain lipids themselves. The CD1 family of membrane determinants are highly conserved, non-polymorphic molecules that are able to present to T lymphocytes microbial and endogenous lipids. It has been reported recently that lipids from pollens, presented via a CD1-dependent pathway, can be recognized by T lymphocytes that could in turn facilitate IgE production [53]. It is possible, but unproven, that some of the lipids derived from Brazil nut might be recognized in similar fashion by responsive T lymphocytes. The speculation is that lipid-driven T lymphocyte responses might enhance and/or modulate concurrent adaptive responses to protein antigens, including in this instance Ber e 1. In addition to receptor-mediated effects there are a number of other potential mechanisms through which the various lipid fractions may be inducing adjuvant effects on IgG and IgE antibody responses. For example, free fatty acids and polar lipids such as PC may have surfactant properties and act as irritants at the site of administration, inducing cytokines that may augment immune responses.

Finally, as part of these investigations partial characterization was made of the isotype distribution of IgG anti-Ber e 1 antibody responses with respect to IgG1 and IgG2a. For some adjuvant lipids there were no substantial differences with respect to induced IgG1:IgG2a ratios. However, in the cases of type (i) glycolipid-rich and class (ii) β -sitosterol, there was evidence for increased IgG1:IgG2a ratios consistent with a more vigorous Th2 cell-type bias. In the case of β -sitosterol, this was against a background of relatively little IgE (one of five mice being IgE responders) expression. It has been reported previously that higher amounts of IL-4 are required to drive IgE synthesis than those that are sufficient for the synthesis of IgG1 [54]. The speculation therefore is that β -sitosterol induces sufficient IL-4 for the expression of IgG1, but not IgE. Effects by Brazil nut lipids on qualitative aspects of immune responses to Ber e 1 require further clarification and confirmation.

In conclusion, these investigations demonstrate clearly that, with respect at least to the major Brazil nut allergen Ber e 1, the elicitation, or at least optimal elicitation, of adaptive immune responses is dependent upon the availability of certain endogenous lipids. These observations have important implications for our understanding of the development of inhalation and food allergy to plant proteins, and for the conduct and interpretation of methods for the prospective identification of sensitizing proteins.

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