

Cross-allergic Reactions to Legumes in Lupin and Fenugreek-Sensitized Mice

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Abstract

Several legumes may induce allergy, and there is extensive serological cross-reactivity among legumes. This cross-reactivity has traditionally been regarded to have limited clinical relevance. However, the introduction of novel legumes to Western countries may have changed this pattern, and in some studies cross-allergy to lupin has been reported in more than 60% of peanut-allergic patients. We wanted to explore cross-reactions among legumes using two newly established mouse models of food allergy. Mice were immunized perorally with fenugreek or lupin with cholera toxin as adjuvant. The mice were challenged with high doses of fenugreek, lupin, peanut or soy, and signs of anaphylactic reactions were observed. Cross-allergic mechanisms were investigated using serum mouse mast cell protease-1 (MMCP-1), antibody responses, immunoblotting and *ex vivo* production of cytokines by spleen cells. Signs of cross-allergy were observed for all the tested legumes in both models. The cross-allergic symptoms were milder and affected fewer mice than the primary allergic responses. The cross-allergy was reflected to a certain extent in the antibody and T-cell responses, but not in serum MMCP-1 levels. Cross-allergy to peanut, soy, fenugreek and lupin was observed in lupin-sensitized and fenugreek-sensitized mice. Differences in serological responses between primary allergy and cross-allergy might be due to mediation through different immune mechanisms or reflect different epitope affinity to IgE. These differences need to be further investigated.

Introduction

Legumes comprise a large group of foods consumed worldwide. Several legumes are allergenic, and peanut is arguably the most important. Legumes like lupin, fenugreek, soy, lentils and others also have the ability to induce clinical allergy [1–3]. The prevalence of legume allergy differs according to different dietary habits and environmental factors around the world [4]. However, the dietary patterns are rapidly changing, and many foods that are traditional in some parts of the world are now being introduced in other parts.

Lupin and fenugreek are examples of two legumes that the general public is increasingly exposed to due to changes in dietary habits. Following the introduction of lupin into processed foods in Northern Europe and Australia in the late 1990's, allergic reactions started to be reported [5–8]. In the beginning, the major part of the reported cases was caused by a cross-allergic reaction in peanut allergic patients, but a literature search by Jappe

and Vieths in 2010 revealed as many cases caused by primary lupin allergy as cases caused by cross-allergy [9]. In 2006, lupin was included in EU's list of allergenic foods that must be declared without exemptions (Directive 2000/13/EC, Annex IIIa). Fenugreek is traditionally used in Indian cooking and is a main ingredient in curry. In 2005, the Norwegian Register and Reporting System for Severe Allergic Reactions to Food (Norwegian Food Allergy Register) received several reports of peanut allergic patients who reacted to Indian dishes where fenugreek was the cause of the reactions [10]. Allergic reactions caused by fenugreek have also been reported in other countries in Europe and Asia [1, 11, 12].

Cross-reactivity occurs when one antibody binds to different allergens due to highly similar epitopes [13]. However, there is some variation with regard to how well antibodies match the epitopes and in the epitope-antibody affinity [14]. It is common to find serological cross-reactivity *in vitro* to several legumes in humans [15–18]; but this will not necessarily lead to clinical

reactions (cross-allergy) in the patients [3, 19]. Regarding lupin, however, there have been reports of up to 68% cross-allergy in peanut allergic patients [15, 20–22]. Recently, fenugreek has also proven to be a potent allergen causing adverse cross-allergic reactions in peanut allergic individuals [10, 18].

It has been shown that allergen-specific IgE can serve as a useful predictor of challenge outcome [23]. Data from the Norwegian Food Allergy Register [24] regarding legumes indicate that peanut is the allergen giving the highest allergen-specific IgE concentration; lupin and fenugreek give intermediary levels, while green pea and soy give the lowest concentration. Correspondingly, the Register has very few reports of adverse reactions caused by green pea or soy, a substantial number of reports regarding lupin and fenugreek, and many regarding peanut. These data show that there is a need to further investigate cross-allergy in legumes. Most of the work performed on legume allergy has focused on peanut as the major allergenic legume, and information on other types of legume allergy is limited [4]. As we previously have established mouse models of lupin and fenugreek allergy [25, 26], we used these models to address the clinical cross-allergy between the four most common allergenic legumes: lupin, fenugreek, peanut and soy. We also assessed different serological and cellular responses to explore possible mechanisms related to the cross-allergic reactions.

Methods

Animals. Female inbred C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME, USA), 5 weeks old at the start of the experiments, were used. Several experiments have been combined in this study and an account of the animals with immunizations and challenges is therefore given in Table 1. Female Sprague-Dawley rats, 150–200 g (Taconic M&B A/S, Ry, Denmark) were used to perform the passive cutaneous anaphylaxis (PCA) tests. The animals were housed, 3–4 mice or two rats per cage, on NESTPAK bedding (Datesand Ltd, Manchester, UK) in type III macrolon cages in filter cabinets (Scantainers), exposed to a 12-hr/12-hr light/dark cycle (30–60 lux in cages), room temperature of $21 \pm 2^\circ\text{C}$ and 35–75% humidity. Pelleted food (RM1; SDS, Essex, UK) and tap water *ad libitum* were given. Before entering the experiments, the animals were allowed to rest for 1 week. The experiments were performed in conformity with the laws and regulations for experiments with live animals in Norway and were approved by the Norwegian Animal Research Authority under the Ministry of Agriculture.

Legume extracts. The National Veterinary Institute of Norway provided all protein extracts. In short, extracts of peanut, lupin and soy were made by extracting homogenized peanuts, soybeans or lupin (*Lupinus angustifolius*) in Tris/glycine buffer, pH 8.7, overnight followed by

Table 1 Account of groups and number of animals in the two models.

Model	Immunization	Challenge	No. of animals	i.p. challenge	p.o. challenge	Trial no.
Lupin	Lupin	Lupin	28	11	17	A, B
		Fenugreek	16	8	8	B
		Peanut	16	8	8	B
		Soy	16	8	8	B
		–	13	–	–	A, B
	CT only	Lupin	4	1	3	A
		–	2	–	–	A
	Naïve	Lupin	4	1	3	A
		–	10	–	–	A, B
		–	–	–	–	–
Fenugreek	Fenugreek	Fenugreek	20	9	11	C, D
		Lupin	16	16	–	C, D
		Peanut	17	17	–	C, D
		Soy	17	17	–	C, D
		–	11	–	–	C
	CT only	Fenugreek	9	5	4	C, D
		–	2	–	–	C
	Naïve	Fenugreek	6	2	4	C
		–	8	–	–	C, D
		–	–	–	–	–

CT, cholera toxin; A, Lupin I; B, Lupin II; C, Fenugreek I; D, Fenugreek II (I and II indicate separate experiments).

centrifugation. The fenugreek extract was made using an extended protocol utilizing precipitation with $(\text{NH}_4)_2\text{SO}_4$, dialysis and freeze-drying [26]. The total protein concentration of the extracts was measured by Lowry's method. The endotoxin level of the extract was determined with the Limulus Amebocyte Lysate (LAL) Kinetic-QCL Kit (BioWhittaker, Walkersville, MD, USA) and found to be below 0.1 ng/ml for all extracts.

Immunizations and challenges. Immunizations were performed perorally (p.o.) according to the experimental protocols previously established [25, 26]. Briefly, immunizations were performed on days 0, 1, 2, 7, 21 and 28 and challenges on day 35. Lupin immunized mice received 5.0 mg lupin protein together with 10 μg cholera toxin (CT, from *Vibrio cholerae*, azide free; EMD Biosciences Inc., CA, USA) per immunization, while fenugreek immunized mice received 4.2 mg fenugreek protein with 10 μg CT per immunization. Blood samples before challenge were obtained from *v. saphena lateralis* on day 33/34.

Challenges were performed with a large dose of one of the protein extracts (peanut, lupin, fenugreek and soy). Based on previous experience, the doses were 25 mg p.o. and 5 mg intraperitoneally (i.p.). Challenge with the primary allergen was carried out by both routes in the two models. In the lupin model, challenge with cross-reactive legumes was performed both p.o. and i.p., but as the responses did not seem to differ with regards to anaphylactic reactions or mast cell responses between the two challenge routes in this model, only i.p. challenges were performed with cross-reactive legumes in the fenugreek model. The p.o. dose was divided into two equal doses given 30 min apart. Some mice were not challenged and

are referred to as immunized only. Control mice were either treated with CT only (sham immunized) or left untreated (naïve mice) (Table 1).

Assessment of clinical anaphylactic reactions. Anaphylactic symptoms were evaluated continuously from the start of the challenge until 30 min after the i.p. challenge or the second p.o. challenge. The scoring system described by Li *et al.* [27] was used: 0 – no symptoms; 1 – scratching and rubbing around the nose and head; 2 – puffiness around the eyes and mouth, diarrhoea, pilar erecti, reduced activity and/or decreased activity with an increased respiratory rate; 3 – wheezing, laboured respiration, cyanosis around the mouth and tail; 4 – no activity after prodding or tremor and convulsion; 5 – death. The mice were exsanguinated immediately after the assessment of the anaphylactic reactions. The clinical anaphylactic reactions were analysed by Ordinal Regression using Statistical Package for Social Sciences (SPSS version 14.0; SPSS Inc., Chicago, IL, USA). Because of a quasi-complete separation in the data, contingency table analysis (Fisher exact test) was used to validate the statistics of the Ordinal Regression.

Serum mouse mast cell protease-1 (MMCP-1) assay. Serum levels of mouse mast cell protease-1 (MMCP-1) were determined at exsanguination with an ELISA kit (Moredun Scientific Ltd., Edinburgh, UK) and performed according to the manufacturer's instructions. Results were analysed by one-way ANOVA on log transformed data, and significant differences between the groups were determined by the Holm-Sidak method. Results are presented as box-plots showing the median, 25th–75th percentile, 10th–90th percentile and outliers.

Total and allergen-specific IgE analyses. Due to the inclusion of several sub-studies (Table 1), sera were analysed for total IgE before (49 mice), after (67 mice) or both before and after challenge (89 mice). Total IgE was determined by sandwich ELISA according to the protocol provided by the manufacturer using Mouse IgE ELISA Sets (Cat. No. 555248; BD Biosciences, San Jose, CA, USA). Statistical testing was performed separately for results before and after challenge. Results are presented as box-plots showing the median, 25th–75th percentile, 10th–90th percentile and outliers.

Lupin- and fenugreek-specific IgE antibodies were determined in individual sera at exsanguination by the heterologous PCA-test [25, 26]. In the lupin model, we also tested for cross-reactivity by the use of the PCA-test, using legume extracts other than lupin. Briefly, mouse serum (100 µl) was injected intradermally in rats. Twenty-four hours later, a saline solution containing legume extract (0.1 mg/ml) and Evans Blue (4.5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was administered i.v. One hour later, the rats were killed and the reactions were read as size in diameter of the blue dots in the skin (illustrations in [25]). All serum samples were diluted 1:4. Prechallenge sera were

not available for PCA because of the relatively large amount of mouse serum needed to perform the test.

IgG1 ELISA. Polystyrene microtiter plates (Maxisorp; VWR International, Radnor, PA, USA) were coated with 2 µg/ml lupin or fenugreek extract and incubated for 2 h at 37 °C and then at 4 °C overnight. Serum samples and antibodies were diluted 1:100 in PBS with 0.05% Tween 20 (PBS-Tw). PBS-Tw was also used as washing buffer between each step. Eight selected serum samples were preincubated with extracts of lupin, fenugreek, peanut, soy or OVA in concentrations from 0 to 10 mg/ml for 1 h to demonstrate the inhibitory potential of the corresponding extracts. All samples were added to the plates and incubated for 2 h at 37 °C. Antibodies were detected by peroxidase-labelled rat monoclonal anti-mouse IgG1 (BD Pharmingen, Franklin Lakes, NJ, USA) for 1 h at 37 °C and peroxidase substrate (ortho-phenylenediamine chloride; Sigma-Aldrich). Absorbance was determined with an ELISA reader (EL808; BioTek Instruments, Winooski, VT, USA) at 450 nm. Antibody concentrations were given in arbitrary units (AU) per ml. Results are presented as a dose-response curve of the median values and box-plots showing median, 25th–75th percentile, 10th–90th percentile and outliers.

Splenocyte preparation. Spleen cells were prepared by pressing the spleens through a 70-µm cell strainer (BD Labware, Franklin Lakes, NJ, USA). The cell concentrations were determined using a Coulter Counter Z1 (Beckman Coulter Inc., Miami, FL, USA). After incubation in culture medium (RPMI 1640 with L-glutamine, supplemented with 10% foetal bovine serum and 1% streptomycin/penicillin) with or without 50 µg/ml legume extract at 37 °C and 5% CO₂ for 5 days, the supernatants were collected and stored at –80 °C awaiting analyses. Trial A (Table 1) was performed to establish the lupin model and was included in the analyses to strengthen the control groups. Spleen cells from mice in this trial were only stimulated with lupin extract.

Ex vivo cytokine production and quantification. The levels of IL-2, IL-4, IL-5, IL-10, IL-13 and IFN-γ in spleen cell supernatants were determined by sandwich ELISA according to protocols provided by the manufacturer. Mouse DuoSets (R&D Systems Inc., Minneapolis, MN, USA) were used. Cytokines were analysed using a Two-way Repeated Measures ANOVA on log-transformed data, and significant differences between the groups were determined by the Holm-Sidak Method. Only results of spleen cells stimulated with all four legumes were included in the statistical analyses, thus excluding trial A. Results are presented as geometric means with 95% confidence intervals.

SDS-PAGE and western blotting. Chemicals and equipment for SDS-PAGE and immunoblot were purchased from Invitrogen unless stated otherwise. The NuPAGE Gel System was used for electrophoretic separation of proteins according to the manufacturer's instructions. In

short, legume extracts and OVA grade VII (Sigma-Aldrich) were diluted in a reducing buffer containing lithium dodecyl sulphate to a concentration of 2–3 mg/ml. The samples were separated for 35 min at 200 V in running buffer (NuPage[®] MES SDS) using NuPage[®] 4–12% Bis-Tris gel and SeeBlue Plus2[®] pre-stained reference standard. The gels were either stained with Simply Blue[™] Safe Stain or electrophoretically transferred onto nitrocellulose membranes (pore size 0.45 µm) in an XCell Blot Module at 30 V and 170 mA for 1 h. Tris-buffered saline (TBS) with Tween20 was used as washing buffer. Skim milk (1%) in TBS was used as blocking and assay buffer.

After 1 h blocking, blots were incubated under gentle shaking overnight at 4 °C with sera from mice immunized with either lupin or fenugreek, or non-immunized, diluted 1:100. All further steps were carried out at room temperature. The blots were incubated successively with two antibodies, first for 1 h with rat anti-mouse IgE (1:1000; Experimental Immunology Unit, University of Louvain, Belgium), and thereafter for 1 h with HRP-conjugated goat anti-rat IgG (1:10 000). TMB substrate solution or ECL Chemiluminescence Substrate (PerkinElmer Inc., Waltham, MA, USA) was used for development. The blots were analysed using Image Lab[™] Software 2.0.1. (Bio-Rad Laboratories Inc., Hercules, CA, USA). In a separate experiment, a selection of sera with high total and specific IgE (lupin or fenugreek) were preincubated with 2 mg/ml of extracts of fenugreek, lupin, peanut or soy for 2 h before incubation of the blots to inhibit the reaction of the corresponding antibodies.

Statistical analysis. Statistical analyses were performed using SigmaStat[®] Statistical Analysis System for Windows Version 3.5 (Systat Software Inc., San Jose, CA, USA) unless otherwise stated. All tests were performed two tailed and differences were considered significant if the p-values were found less or equal to 0.05. Apart from cytokines and MMCP-1, data could not be transformed to a normal distribution, and the non-parametric Kruskal-Wallis Analysis of Variance on Ranks was used. Pair wise comparisons were carried out by Dunn's method to account for unequal group sizes. A two-way ANOVA was performed to assess differences between the routes of challenge regarding MMCP-1, while Fisher exact tests were used to address this regarding anaphylaxis. Results were pooled for subsequent analyses when no differences between i.p. and p.o. challenge or interactions could be found.

Results

Anaphylactic reactions were observed after challenge with all four legumes

In the lupin model, close to 70% of sensitized mice challenged with lupin developed reactions with a score

of 2 or higher (Table 2). Challenged with peanut, soy or fenugreek 37.5%, 31.5% and 12.5% of the lupin-sensitized mice developed serious anaphylaxis (score 2 or higher), respectively. Twenty-five percent of the fenugreek challenged mice did not react, while all sensitized mice challenged with peanut or soy showed at least a weak reaction with increased itching. All sensitized groups showed significantly higher anaphylactic score compared to control groups ($P < 0.001$), and the lupin challenged mice showed significantly stronger reactions than mice challenged with soy ($P = 0.004$), peanut ($P = 0.01$) and fenugreek ($P < 0.001$) (Fig. 1A). I.p. challenge with lupin resulted in more serious reactions than p.o. challenge with lupin, but no differences could be seen regarding route of challenge (i.p. versus p.o.) for the cross-allergens (peanut, soy and fenugreek).

In the fenugreek model, all sensitized mice challenged with fenugreek developed serious anaphylactic reactions of score 2 or higher (Table 2). Mice challenged with fenugreek i.p. developed more serious reactions than mice challenged with fenugreek p.o. Challenged with peanut or soy about 30% of the fenugreek-sensitized mice developed serious reactions, while 75% of lupin challenged mice reacted with a score of 2 or more. Peanut and soy challenges did not result in any clinical reaction in 37.5% and 31.25% of the mice, respectively, while all mice except one reacted to lupin. All sensitized groups showed significantly higher anaphylactic score than control groups ($P < 0.001$ for fenugreek, i.p. and p.o., and lupin; $P = 0.02$ for soy and peanut), and the fenugreek challenged mice showed significantly stronger reactions than mice challenged with lupin, soy or peanut ($P \leq 0.001$) (Fig. 1C).

Intestinal anaphylaxis by MMCP-1 reflected primary allergic responses

MMCP-1 was measured as a reflection on the local anaphylactic reaction in the gut, as it is released from mast cells upon activation. Sensitized mice challenged

Table 2 Account of anaphylactic reactions in the two models.

Model	Challenge	Score ≥ 1 (%)	Score ≥ 2 (%)
Lupin	Lupin	28/28 (100)	19/28 (67.9)
	Peanut	16/16 (100)	6/16 (37.5)
	Soy	16/16 (100)	5/16 (31.5)
	Fenugreek	12/16 (75)	2/16 (12.5)
	Controls	0/8 (0)	0/8 (0)
Fenugreek	Fenugreek	20/20 (100)	20/20 (100)
	Lupin	15/16 (93.8)	12/16 (75)
	Soy	11/16 (68.8)	5/16 (31.5)
	Peanut	11/17 (64.7)	5/17 (29.4)
	Controls	1/15 (6.7)	0/15 (0)

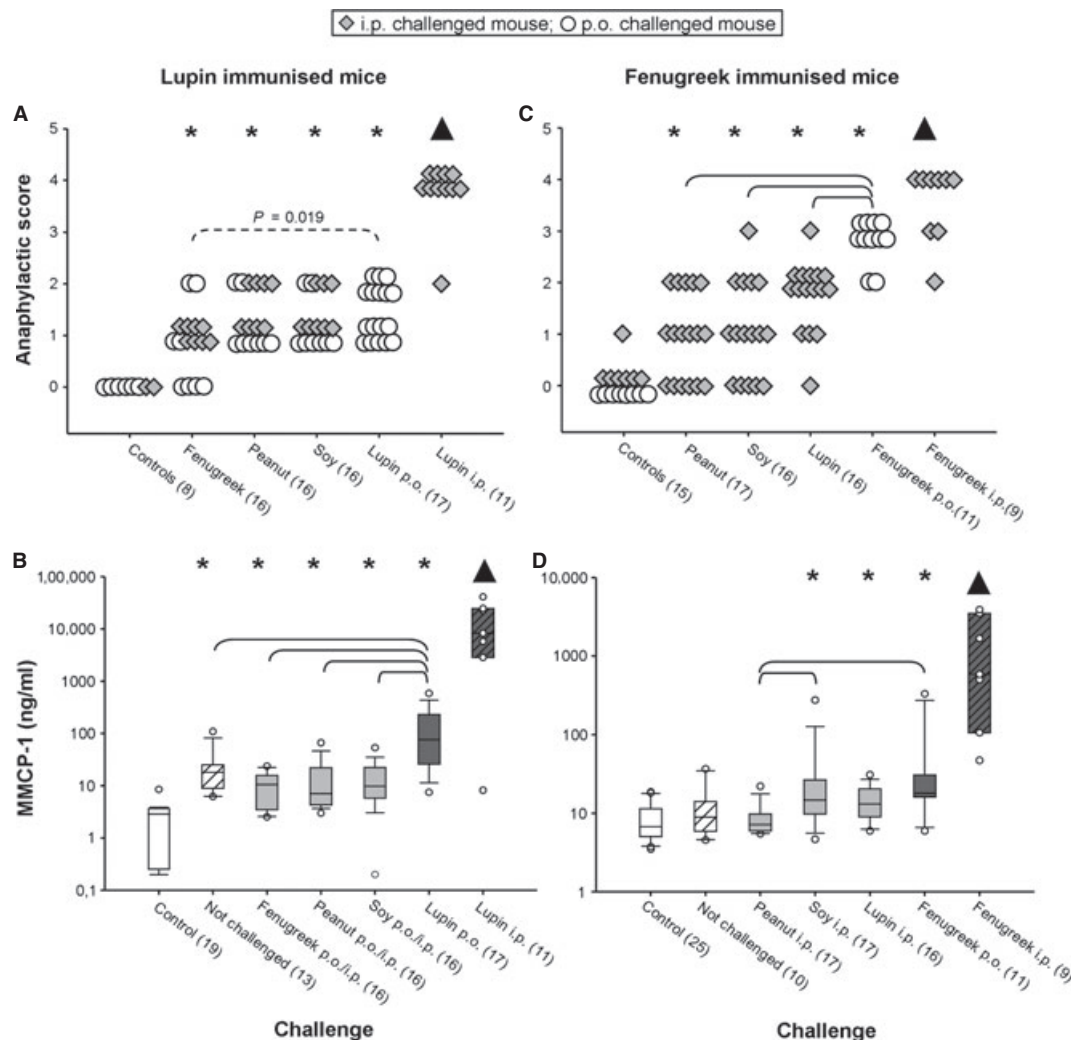


Figure 1 Clinical symptom scores in (A) lupin immunized mice and (C) fenugreek immunized mice after challenge with high doses of the indicated legume extracts. Each circle/diamond represents one animal. White circles denote p.o. challenged mice and grey diamonds denote i.p. challenged mice. The intestinal anaphylaxis as measured by MMCP-1 in (B) lupin immunized mice and (D) fenugreek immunized mice was determined in serum after exsanguination. The box plots show median, 25th–75th percentile, 10th–90th percentile and outliers. Overall, P -value of the ANOVA was <0.001 . ▲Significantly higher than all other groups, and *significant difference to controls. Brackets indicate significant differences between groups. Controls are sham immunized and naïve mice combined. Group sizes are indicated in parentheses.

i.p. with the primary antigen responded with a MMCP-1 level much higher than all other groups in both models, including mice challenged p.o. with the primary allergen (Fig. 1B,D). In contrast, mice challenged p.o. with lupin in the lupin model (Fig. 1B) had higher MMCP-1 levels than the other groups, while mice challenged (both p.o. and i.p.) with peanut, soy or fenugreek as well as only immunized mice (not challenged) all had significantly higher levels than control mice. No differences could be seen between p.o. and i.p. challenge regarding the cross-allergens (peanut, soy and fenugreek). Mice challenged p.o. with fenugreek and i.p. with soy in the fenugreek model (Fig. 1D) showed significantly higher MMCP-1 levels than controls and peanut challenged mice, while fenugreek-sensitized mice challenged with lupin showed

higher levels than the controls only. Peanut challenged mice and unchallenged mice did not show significantly higher levels than control mice.

In summary, mice challenged with the primary allergen displayed significantly higher levels of MMCP-1 than the other groups. Mice challenged with a potentially cross-reactive allergen showed higher levels of MMCP-1 than control mice, however, the levels were comparable with mice that were only immunized and not challenged. There was a significant correlation between the anaphylaxis score and MMCP-1 with a Spearman's ρ rank correlation coefficient of 0.417 for the lupin model, 0.448 for the fenugreek model and 0.409 for both models combined, $P \leq 0.001$.

Cross-allergic reactions may be mediated by both IgE and IgG1

The involvement of IgE in the cross-allergic reactions was studied with different methods in the two models. In the lupin model, we used the PCA-test to investigate possible cross-reactions by injecting legumes other than lupin *i.v.* but no reactions could be observed in this test. In the fenugreek model, total IgE was measured in all mice both before and after challenge (Fig. 2A). Comparing total IgE levels before and after challenge in each group according to allergen challenge (*t*-test) revealed significant differences in fenugreek challenged mice ($P = 0.002$), peanut challenged mice ($P = 0.039$) and lupin challenged mice ($P = 0.047$), but not in soy challenged mice. Correspondingly, in the analysis of the groups before challenge, all groups had higher IgE levels than control mice, while total IgE levels after challenge with fenugreek, peanut or lupin were not significantly different from the controls.

In Western blotting (Fig. 2B), we were only able to detect IgE binding to lupin in sera from mice immunized with lupin, where several IgE binding bands were revealed in the range from about 50 kDa to about 70 kDa. These sera also showed binding to a fenugreek band of approximately 50 kDa (Fig. 2B, arrow) and a band of approximately 60 kDa. As the latter band also could be seen with sera from naïve mice, this is presumably unspecific binding that might be due to the presence of lectin in the extract. Mice immunized with fenugreek showed IgE binding to fenugreek only, with several bands revealed between 50 kDa and about 150 kDa. No binding to peanut, soy or OVA was detected in any of the blots (not shown). Preincubation with the primary allergen inhibited all IgE binding, while potentially cross-reacting allergens did not inhibit the IgE binding substantially (not shown).

Immunized mice showed high levels of IgG1 that were completely inhibited by preincubation with the primary allergen in both models (Fig. 3). In the lupin model (Fig. 3A,B) a partial inhibition could also be seen after incubation of lupin positive sera with extracts of peanut and soy at the highest concentration (10 mg/ml). In the fenugreek model (Fig. 3C,D) only peanut displayed a partial inhibition of fenugreek positive sera at this concentration.

In general, all antibody reactions, total and specific IgE as well as specific IgG1, were elevated in immunized animals compared to control groups, regardless of challenge (Figs 2 and 3).

Cytokine release may reflect some of the cross-allergic reactions

Fenugreek had an inhibitory effect on the levels of all cytokines in both models both *in vivo*, after challenge,

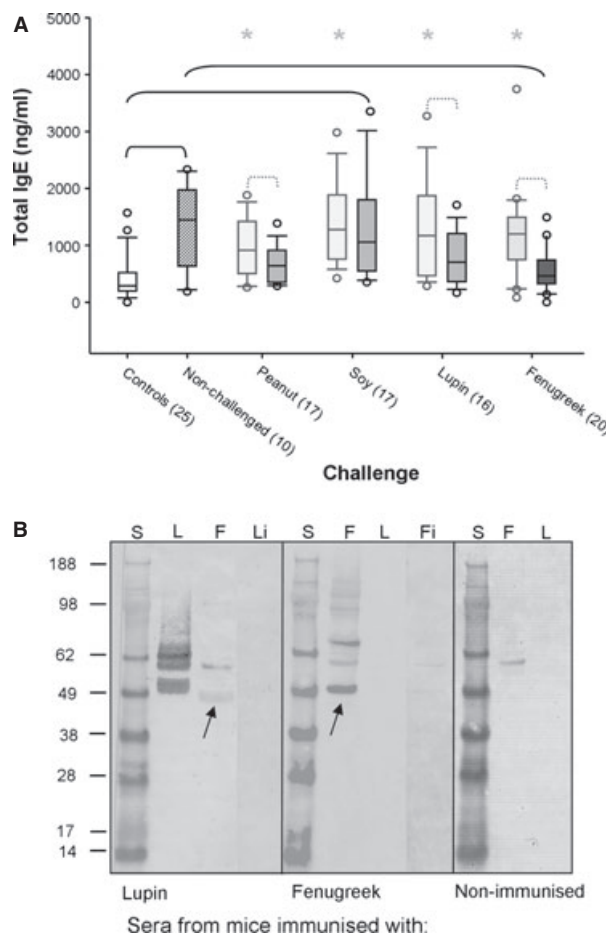


Figure 2 (A) Total IgE before and after challenge in fenugreek immunized mice. Total IgE was not measured in the lupin immunized mice before challenge, and therefore only results from fenugreek immunized mice are shown. Group sizes are indicated in parentheses. The box plots show median, 25th–75th percentile, 10th–90th percentile and outliers. Grey boxes show levels before challenge and black boxes after challenge. *Significant difference to controls. Brackets indicate significant differences between groups. Dotted grey brackets indicate significant difference before and after challenge within the same group using the *t*-test. Controls are sham immunized and naïve mice combined. (B) Representative western blots using sera from mice immunized with lupin or fenugreek and non-immunized mice. The arrow points at the cross-reactive fenugreek protein of about 50 kDa. S-lanes – Molecular weight ladder (SeeBlue® Plus2 PreStained Standard); L-lanes – lupin extract; F-lanes – fenugreek extract; i – serum has been preincubated with extract to show inhibition. Protein sizes (kDa) are indicated on the right side of the blots.

and *ex vivo*, after spleen cell stimulation (Fig. 4, IL-4 and IL-13; and supplementary figure (Fig. S1), IL-2, IL-5, IL-10 and IFN- γ). This is reflected by lower cytokine levels in spleen cells from fenugreek immunized mice when stimulated with fenugreek compared to cells stimulated with lupin. In both models, stimulation with the primary allergen yielded strong responses with a mixed Th1/Th2 profile, but with an emphasis on Th2 responses, as reported earlier [25, 26]. A positive cytokine response was defined as a response significantly higher

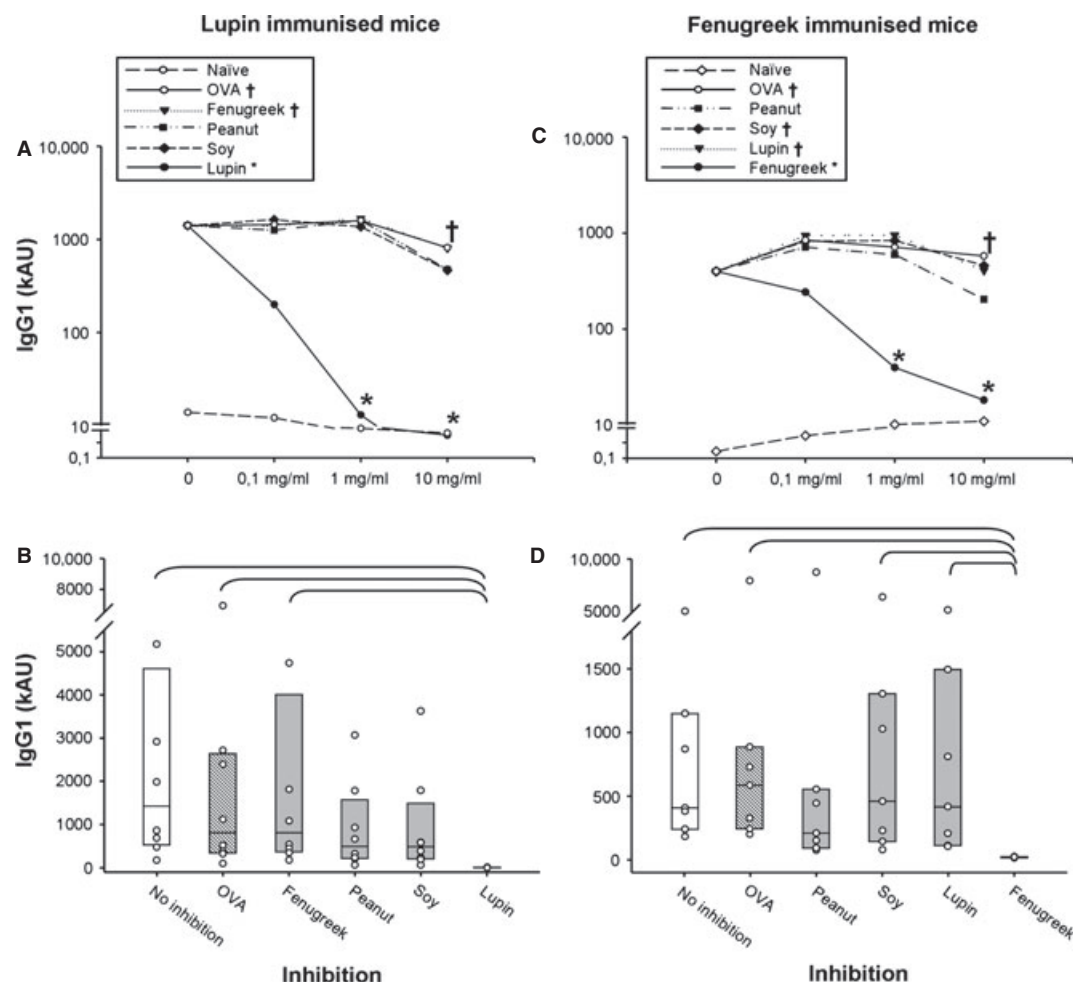


Figure 3 Inhibitory effect of preincubation of eight selected sera with legume extracts and OVA is shown. (A, B) Lupin specific IgG1 and (C, D) fenugreek specific IgG1 measured by ELISA. The top panels (A, C) show a dose-response effect of the inhibition indicating median values, while the bottom panels (B, D) show box-plots with median, 25th–75th percentile, 10th–90th percentile and outliers (circles) at the highest inhibitory dose (10 mg/ml). *Significant differences from no inhibition; †Significant differences from inhibition with the homologue antigen at the highest inhibitory dose (10 mg/ml). Brackets indicate significant differences between groups.

than the cytokine release from unstimulated cells and significantly higher than cytokine release from cells of control animals stimulated with the same allergen.

When looking at the responses after stimulation with cross-allergens in the model of lupin allergy, stimulation with soy extract yielded higher IL-4 and IL-13 responses compared to unstimulated cells and control cells stimulated with soy (Fig. 4A,B). Peanut stimulated cells from mice challenged with lupin also released higher levels of the same cytokines, however only significantly higher than unstimulated cells and not to peanut stimulated control cells.

In the model of fenugreek allergy, the inhibitory effect of fenugreek on the spleen cells both *in vivo* and *ex vivo* makes it difficult to evaluate possible cross-reactions. There is, however, a tendency to increased responses after lupin stimulation regarding IL-2, IL-4 and IL-10 when

compared to unstimulated cells, but no differences could be seen between the different groups of mice (Fig. 4C,D).

Discussion

In two mouse models of legume allergy, we have shown clinically relevant cross-allergy to other legumes. The proportion of cross-allergy in sensitized mice varied from 12.5% up to 75% with a clinical score of 2 or higher. The majority of the legumes displayed a cross-allergy of 30% or more. This is in contrast to Lifrani *et al.* [28] who demonstrated cross-reactivity *in vitro* between peanut and lupin, but could not find any cross-allergy to lupin in peanut sensitized mice. Our finding is, however, in concordance with findings from the Norwegian Food Allergy Register [24] and other publications on cross-allergy to lupin [15, 19–22, 29] and fenugreek in

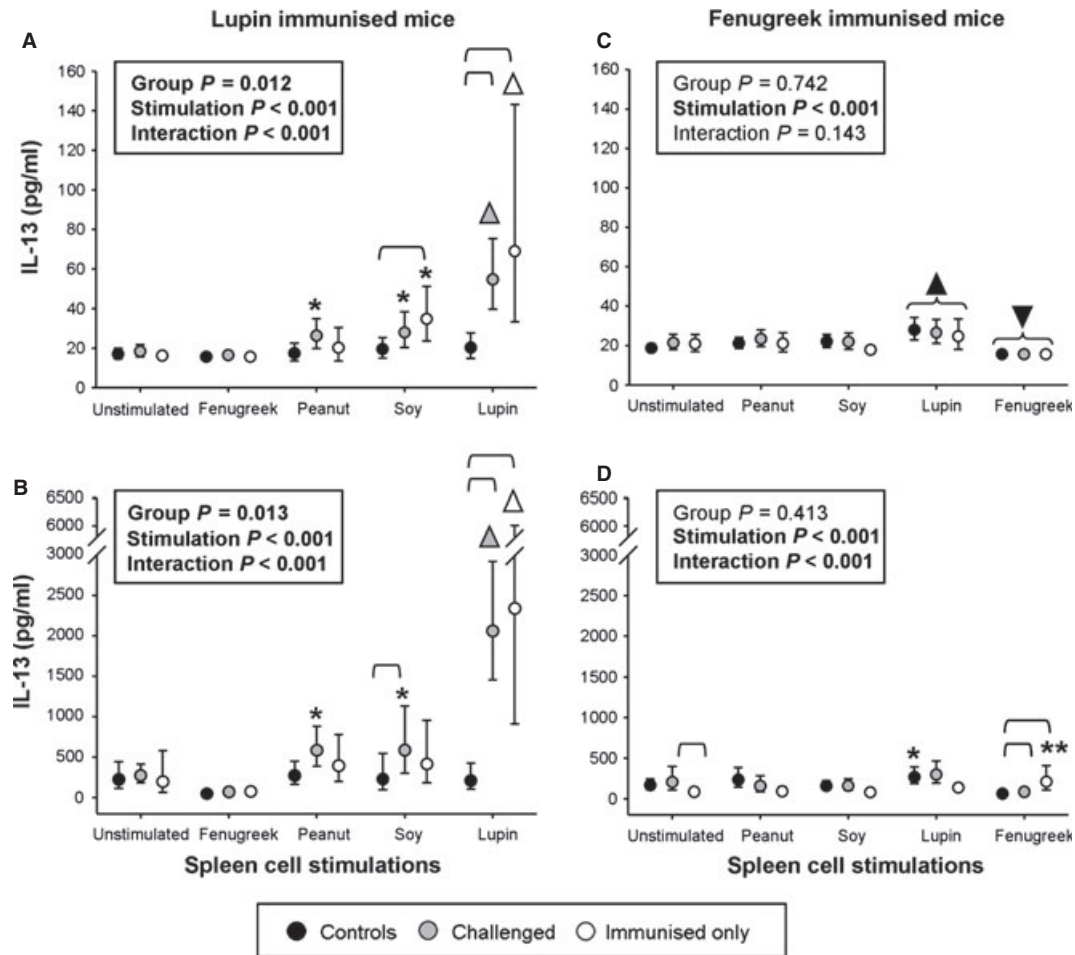


Figure 4 Cytokines were measured in supernatants from spleen cell cultures from mice challenged with the homologous extract according to immunization (grey circles; $n = 16$ in the lupin model and $n = 20$ in the fenugreek model), mice that were immunized, but not challenged (white circles; $n = 7$ in the lupin model and $n = 10$ in the fenugreek model) and controls (black circles; $n = 8$ in the lupin model and $n = 25$ in the fenugreek model). Cells from each spleen were incubated with extracts of lupin, fenugreek, peanut and soy, and in medium (unstimulated). Results are presented as geometric means with 95% confidence intervals. Overall P -values are given in the boxes, with statistically significant values in bold. Brackets indicate significant differences in the *post-hoc* tests between cell treatments in each group according to immunization status ($P < 0.05$). Triangles pointed up denote significantly higher levels than the other stimulations within the same group. Triangles pointed down denote significantly lower levels than the other stimulations within the same group. *denotes significantly higher levels than unstimulated cells within the same group, and **denotes significantly higher levels than unstimulated, soy stimulated and peanut stimulated cells (D only). Only differences important to possible cross-reactivity are shown.

peanut-sensitized individuals [10]. This illustrates the potential for cross-allergy in legume allergic patients, even though this has earlier been regarded as relatively rare [30, 31]. In line with observations in humans, the cross-allergic reactions observed in the present study were less severe and affected fewer animals than the primary allergy.

Serum MMCP-1 has been shown to be a marker for mucosal mastocytosis and increased gut permeability [32] as well as for mast cell dependent intestinal inflammation [33]. A strong correlation between anaphylactic score and levels of MMCP-1 was found. However, cross-allergy did not reveal any signs of mast

cell activation, as the levels of MMCP-1 in animals challenged with cross-reactive legumes were comparable with the levels of immunized, not challenged animals. This suggests that intestinal mast cells are less activated in the cross-allergic reactions observed. It has been reported that food induced anaphylaxis may depend more on macrophages and basophils than on mast cells [34], and more studies are needed to elucidate the roles of macrophages and basophils in cross-allergy. That no cross-reactivity could be observed in the PCA-test may also support the notion that cross-allergic reactions are not mediated through a mast cell dependent pathway. However, because of the functionality of the test, it

could also be a reflection of the difference in affinity between epitopes.

Two distinct mechanisms have been reported to induce systemic anaphylaxis in the mouse [35]. The classical pathway is mediated by allergen cross linking of IgE bound to the high affinity receptor (Fc ϵ RI) on mast cells. The alternative pathway is thought to involve macrophages, Fc γ RIII, IgG antibodies and platelet activating factor [36]. A partial inhibition of lupin specific IgG1 by peanut and soy and of fenugreek specific IgG1 by peanut was observed. A role for both IgE and IgG1 in the cross-allergic responses in mice is therefore possible. Several studies have implied that both the classical and the alternative pathway of food induced anaphylaxis are involved simultaneously in mice, and that abrogation of one pathway only partially abrogates anaphylactic responses [37–39]. Tsujimura *et al.* [40] demonstrated that basophils play a crucial role in IgG mediated anaphylaxis in their mouse model. It has also been reported that mast cells contribute to anaphylaxis through both IgE and IgG1, whereas macrophages contribute through IgG1 exclusively. The role of IgG1 in anaphylactic reactions in mice complicates the extrapolation of findings from mouse to man, as IgG-mediated anaphylaxis to food has not yet been described in man. The relevance to human anaphylaxis of the different pathways observed in mice needs to be investigated. Strait *et al.* have shown that although the IgE pathway is more sensitive and requires lower threshold levels of antigen for full activation, IgG mediated responses can also be severe [36, 41]. Our studies support the involvement of IgG1 in cross-allergy, while we were unable to confirm the involvement of IgE and mast cells. The precise mechanisms underlying the pathogenesis of food allergy remain to be fully elucidated. Based on our data, it is tempting to speculate that there is a difference in the mechanisms underlying cross-allergy compared to primary allergic reactions. In our mouse models, the cross-allergy seems to depend on a combined IgE and IgG1 mediated pathway, while the primary allergy seems to be IgE and mast cell dependent.

Studies in human patients have shown differences in measurable cross-reactivity between skin-prick tests and Western blotting [16, 20, 42]. This may be explained by differences in epitope and antibody affinity requirements as well as test sensitivity. Clinical and humoral responses in our models also showed some differences. Clinically, all legumes caused some degree of cross-allergy. Serological responses, however, differed according to the primary sensitization and the laboratory test. While no cross-reactivity could be observed by Western blotting in the fenugreek model, IgE binding to fenugreek was detected in lupin sensitized mice. The 50 kDa fenugreek band has been characterized by Faeste *et al.* [43] as a 7S globulin with the proposed name Tri f1, a homologue to the major allergens Ara h1 in peanut, Lup an 1 in lupin and

Gly m 5 in soy [44–46]. It has been reported that different allergens need different doses to inhibit responses in Western blotting [47], which may correspond to different affinity of the cross-reacting epitopes to IgE. Partial denaturation and loss of some crucial allergens from the blots might also be an explanation, although the known relevant bands appeared to be present. Total IgE measured before and after challenge indicated IgE mediated cross-reactivity to peanut and lupin in the fenugreek model as we observed a fall in total IgE upon challenge [26]. However, this fall might also be caused by increased vascular leakage during anaphylaxis.

In general, cytokine release after spleen cell stimulation is a reflection of T cell responses, and in the characterization of the two models we have demonstrated that the primary allergens promote a Th2 response [25, 26]. However, the cytokines IL-4 and IL-13 play important roles in both the induction and effector phases of allergic responses. In the lupin model, signs of cross-reactivity could be seen after stimulation with soy and peanut on the release of IL-4 and IL-13. T cells recognize small peptides that have been processed and presented to them on the MHC-II molecules by antigen presenting cells during the sensitization. IgE antibodies, on the other hand recognize larger, conformational epitopes on the surface of the intact protein, and the epitope specificity on the T cell level is different from the epitope specificity on the antibody level. Cross-allergy is defined by antibody binding, while T cells mainly are involved in the sensitization phase of the reaction. T cell specificity could thus be seen as irrelevant to the clinical reactions. However, the proteins responsible for the cross-reactivity are proteins that are highly conserved, and cross-reactivity at the T cell level might therefore be a strong predictor for cross-allergy at the antibody level. Further, the role of T cells in the allergic reaction has been little explored, but T cells together with eosinophils have been regarded to be important for the late phase reaction [48]. Fenugreek exhibits properties that are inhibitory on all cytokine release, an effect evident both after *in vivo* and *ex vivo* exposure and in both models. The inhibitory effect on cytokines is in accordance with the suggested immunomodulatory properties of fenugreek [49, 50]. This makes it difficult to draw any conclusions based on the cytokine profile in the fenugreek model.

In allergy testing of humans the outcome may be that a number of IgE mediated serological reactions occur with no apparent clinical relevance [23, 51, 52]. In contrast, our mouse models showed clinical reactions sometimes without correlating serological responses, an event rarely seen in man. This could be related to the sensitivity or relevance of the laboratory tests, or it could be an expression of differences between man and mice, including a difference in allergen exposure. Mice live in a very controlled and sheltered environment and are essentially

exposed to only one legume, the experimental one. Humans, on the other hand, are exposed to several different legumes from early on in life, which makes co-sensitization a possible cause of apparent cross-allergic reactions [13, 52]. In the mouse models, we essentially have mono-sensitization and the observed reactions are thus true cross-allergic responses.

In conclusion, we have in the mouse models shown clinically relevant cross-allergy between the four allergenic legumes, lupin, fenugreek, peanut and soy, reflected to some extent in serological and cellular tests. The effector immune mechanisms underlying cross-allergic reactions in mice and their relevance for man still remain to be fully elucidated. Our models may prove valuable for the study of cross-allergy mechanisms and the role of individual allergen components.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Figure S1 IL-5, IL-10, IFN- γ and IL-2 responses in the two models are shown. Cytokines were measured in supernatants from spleen cell cultures from mice challenged with the homologous extract according to immunization (grey circles; $n = 16$ in the lupin model and $n = 20$ in the fenugreek model), mice that were immunized, but not challenged (white circles; $n = 7$ in the lupin model and $n = 10$ in the fenugreek model) and controls (black circles; $n = 8$ in the lupin model and $n = 25$ in the fenugreek model). Cells from each spleen were incubated with extract of lupin, fenugreek, peanut and soy, and in medium (unstimulated). Results are presented as geometric means with 95% confidence intervals. Overall p-values are given in the boxes, with statistically significant values in bold. Brackets indicate significant differences in the post-hoc tests between cell treatments in each group according to immunization status ($p < 0.05$). Triangles pointed up denote significantly higher levels than the other stimulations within the same group. Triangles pointed down denote significantly lower levels than the other stimulations within the same group. * denotes significantly higher levels than unstimulated cells within the same group, and ** denotes significantly higher levels than fenugreek stimulated and peanut stimulated cells (a only). Only differences important to possible cross-reactivity are shown.

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Supplementary figure

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