

Anaphylactic Reactions in Mice with Fenugreek Allergy

N. E. Vinje*†, E. Namork* & M. Løvik*‡

*Department of Environmental Immunology, Division of Environmental Medicine, Norwegian Institute of Public Health, Oslo, Norway; †Norwegian Veterinary Institute, Oslo, Norway; and ‡Department of Cancer Research and Molecular Medicine, Faculty of Medicine, Norwegian University of Science and Technology, Trondheim, Norway

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Correspondence to: N. E. Vinje, Norwegian Institute of Public Health, Division of Environmental Medicine, Department of Environmental Immunology, P.O. Box 4404 Nydalen, NO-0403 Oslo, Norway. E-mail: nina.eriksen.vinje@fhi.no

Abstract

Fenugreek is a legume mostly used as a spice in Indian-style cooking. Although it has been used since ancient times, its allergenicity has only been reported in the last two decades. It poses special problems as an emerging and often hidden allergen. Fenugreek exposure may have serious implications also for individuals with peanut allergy because of cross-reactivity. Because a new food requires a model specially designed for that particular food, the aim of our study was to develop a food allergy model of fenugreek in mice with anaphylaxis as the endpoint. Mice were immunized perorally using cholera toxin as adjuvant. A two-compartment response surface design with immunoglobulin (Ig)E as the main variable was used to estimate the optimal sensitizing dose of fenugreek, which was further used to evaluate the model. The mice were challenged perorally with a high dose of fenugreek, and signs of anaphylactic reactions were observed. Challenged mice showed high levels of mouse mast cell protease-1, developed specific IgE against several proteins in the fenugreek extract, had elevated levels of IgG1 and IgG2a and showed a general shift towards a Th2 response as determined by *ex vivo* production of cytokines. Total IgE levels were substantially decreased after challenge. In conclusion, we have established a mouse model of IgE-mediated fenugreek allergy demonstrating anaphylactic reactions upon challenge. There is little information on fenugreek cross-allergy to other legumes like peanut, soy and lupin, and we expect that this model will be a valuable tool in further research on legume allergy.

Introduction

Legumes like peanut, soy and lupin are among the major food allergens with significant public health relevance. Peanut is the most common allergen in several European countries [1–3] and the United States [4, 5], and the prevalence of peanut allergy is rising, especially in children [3, 5]. There is extensive serological cross-reactivity among legumes [6]. However, in some cases also clinical cross-reactions between legumes can occur, in particular secondary to peanut allergy, posing a serious problem when the cross-reacting legume is a hidden ingredient in the food.

Fenugreek (*Trigonella foenum-graecum* L.) is a member of the legume family. The aromatic fenugreek seeds can be roasted, ground to a powder and used as an ingredient in curries, chutneys and teas [7]. The plant has a wide variety of therapeutic properties [8] and is used as a traditional functional food. Most studies on fenugreek have focused on the medicinal effects of the plant [8, 9]. Since

the 1990s, however, cases of patients allergic to fenugreek have been reported both in Asia and in Europe [10–13], and recently several potential allergens in fenugreek have been characterized [14]. The potential cross-reactivity of fenugreek to peanut is of great concern, but fenugreek may also be the cause of primary allergy. Fenugreek poses a problem as a commonly hidden and relatively unknown allergen. It first came into attention in Europe in 2006 after the Norwegian Register and Reporting System for Severe Allergic Reactions to Food (The Norwegian Food Allergy Register) received a number of reports of adverse reactions to food containing curry powder. Clinical cross-reactivity between fenugreek and peanut was confirmed in two peanut allergic patients by open food challenge [13]. A Norwegian study of peanut allergic individuals also indicated that fenugreek gives stronger serological cross-reactions than other legumes [15]. In Western cultures, consumption of fenugreek has been limited, but recent trends have made food from India and the Middle East more popular. Thus, we

may expect to see more cases of adverse reactions caused by fenugreek.

Mice have a well-characterized immune system that closely parallels the human immune system. Furthermore, mouse models offer the advantage of studying an immune reaction in its full complexity *in vivo* [16–18]. We wanted to establish a mouse model of fenugreek allergy based on the cholera toxin (CT) model. This has been a successful model of allergy to cow's milk [19], peanut [20], buckwheat [21] and lupin [22]. As every food allergen has unique qualities, the method must be carefully developed and adjusted to each allergen. Most published models have focused on the allergens responsible for the majority of allergic reactions, like milk, peanuts and tree nuts. Fenugreek is a novel and expanding food in the western world and is such comparable to lupin, an allergen recently added to the EU list of allergens that must be declared without exemptions (Directive 2000/13/EC, Annex IIIa). A model of fenugreek allergy will, in addition, be helpful for the understanding of cross-reactions caused by peanut allergy.

We report here the development of an anaphylactic fenugreek allergy model with peroral immunization and clinical symptoms as an endpoint.

Materials and methods

Animals. Female inbred C3H/HeJ mice (Jackson Laboratories, USA), 5 weeks old, were used. Thirty mice were required in the dose–response experiment and 47 in the acute anaphylaxis experiment. A total of 16 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, England, UK) in type III macrolon cages in filter cabinets (Scantainers), exposed to a 12-h/12-h light/dark cycle (30–60 lux in cages), room temperature (RT) of $21 \pm 2^\circ\text{C}$ and 35–75% humidity. Pelleted food (RM1; SDS, Ussex, England, 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.

Fenugreek extraction. The Norwegian Veterinary Institute provided the fenugreek extract. The extract was made as described by Faeste *et al.* [13]. Briefly, fenugreek seeds were extracted in Tris/glycine buffer, pH 8.7, overnight, centrifuged, diluted in distilled water and precipitated with $(\text{NH}_4)_2\text{SO}_4$. After another centrifugation, the pellet was dissolved in Tris buffer, pH 8.0, and filtered

through glass wool. The filtered solution was dialysed against water in a 6- to 8-kD-pore size dialysis tube (Spectra/Por; Spectrum Medical Industries, Los Angeles, CA, USA) and freeze-dried. The freeze-dried powder was dissolved in PBS, and the total protein content was determined to be 36.8 mg/ml by Lowry's method [23]. The endotoxin level of the extract was determined to be 0.0146 ng/ml by the Limulus Amebocyte Lysate (LAL) Kinetic-QCL kit (BioWhittaker, Walkersville, MD, USA). Extracts of peanut, soy and lupin used in SDS–PAGE were made similarly.

Immunizations and challenges. Immunizations were performed by gavage (p.o.) according to an experimental protocol previously published [22]. First, a dose–response experiment (Fig. 1A) and secondly an acute anaphylaxis experiment with the determined dose of fenugreek (Fig. 1B) was performed. In the acute anaphylaxis experiment, there were three main groups of animals (Table 1). The immunized group of mice was immunized gavage with fenugreek and CT, but not challenged. Challenged mice were immunized in the same way and challenged either by intraperitoneal injection (i.p.) or by gavage. Control mice were either treated with CT only (sham-immunized) or left untreated (naïve mice). CT (from *Vibrio cholerae*, azide free) was purchased from EMD Biosciences Inc., San Diego, CA, USA.

The dose–response experiment. The dose–response experiment was performed as a two-compartment response surface design [24] with 15 mice in each compartment. Each mouse received an individual dose of fenugreek together with 10 μg CT. In the first compartment, the doses were equally distributed from 0.1 up to 10.0 mg. Fenugreek immunizations were performed on days 0, 1, 2, 7 and 21, and all mice were exsanguinated on day 28 (Fig. 1A). Fenugreek-specific immunoglobulin (Ig)E as determined by PCA was used as outcome. A PCA reaction of at least 5 mm was used as an indicator of a substantial response. The results from the first part (compartment) were plotted in a graph using dosage (mg/mouse) as the *x*-axis and the size of the PCA reac-

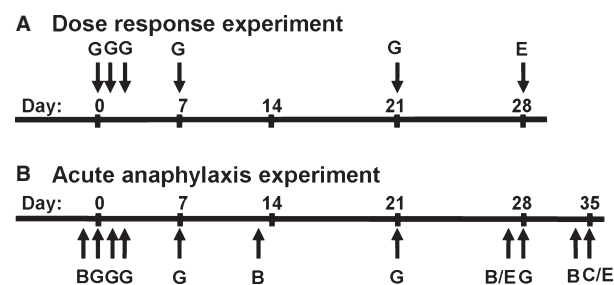


Figure 1 Experimental design. Time lines for the experiments and treatments are given. (A) The dose–response experiment ($N = 30$ mice) and (B) the acute anaphylaxis experiment ($N = 47$ mice). G denotes intragastric gavage, B denotes blood sampling, E denotes exsanguination and C denotes allergen challenge.

Table 1 Experimental groups in the acute anaphylaxis experiment (Fig. 1B).

Groups	Treatment	Challenge	Number of animals	Day of exsanguination
Challenged	Fenugreek + CT	i.p.	6	35
		p.o.	11	35
Immunized	Fenugreek + CT	None	11	27
		i.p.	2	35
Sham controls	CT only	p.o.	4	35
		None	2	27
		i.p.	2	35
		p.o.	4	35
Naïve controls	Not treated	None	2	27
		i.p.	2	35
		p.o.	4	35

CT, cholera toxin.

tion (mm) as the y -axis (Fig. 2A). Non-linear regression analysis with the size of reaction as the dependent variable was used to estimate the doses of fenugreek giving elevated fenugreek-specific IgE responses (JMP[®] 5.0.1a statistical software; SAS Institute Inc., Cary, NC, USA). A polynomial line of second degree (y_A) was determined by the data points, as shown in Fig. 2A.

In the second compartment, the doses of fenugreek were clustered around three focus points determined in the first compartment: the maximum (y_{Amax}) of the curve and the two intersections between the curve (y_A) and the line $y = 5$. The doses in the second compartment ranged from 0.1 up to 6.5 mg. Results from both compartments were then plotted in one graph (Fig. 2B). The same analysis as described above was used to determine the optimal dose of fenugreek extract. The maximum (y_{Bmax}) of the curve indicates the dose of fenugreek extract that statistically should result in the largest PCA reaction and hence the highest IgE response.

The total data set was again analysed by the same type of statistical model, but now with the dosage of fenugreek as the dependent variable. Figure 2C shows the approximation of the 95% confidence interval of the optimal dose, found by exchanging the y -axis with the x -axis from plot B and calculating a new polynomial line (y_C) with confidence intervals (thin lines).

The acute anaphylaxis experiment. In the acute anaphylaxis experiment (Table 1), mice were treated perorally with 4.2 mg fenugreek extract and 10 μ g CT per immunization on days 0, 1, 2, 7 and 21 (Fig. 1B). On day 27, one mouse from each cage was exsanguinated (the immunized group). Remaining mice received an additional immunization on day 28. Blood samples were obtained on days- 1, 13, 27 and 34 from *v. saphena* [25, 26]. The anaphylactic response after challenge was determined on day 35. Eleven fenugreek-immunized mice (p.o. challenged group) and eight control mice (four sham and four naïve) were challenged perorally with a total dose of 25.0 mg fenugreek. The oral dose was divided into two equal doses given 30 min apart. Six fenugreek-immu-

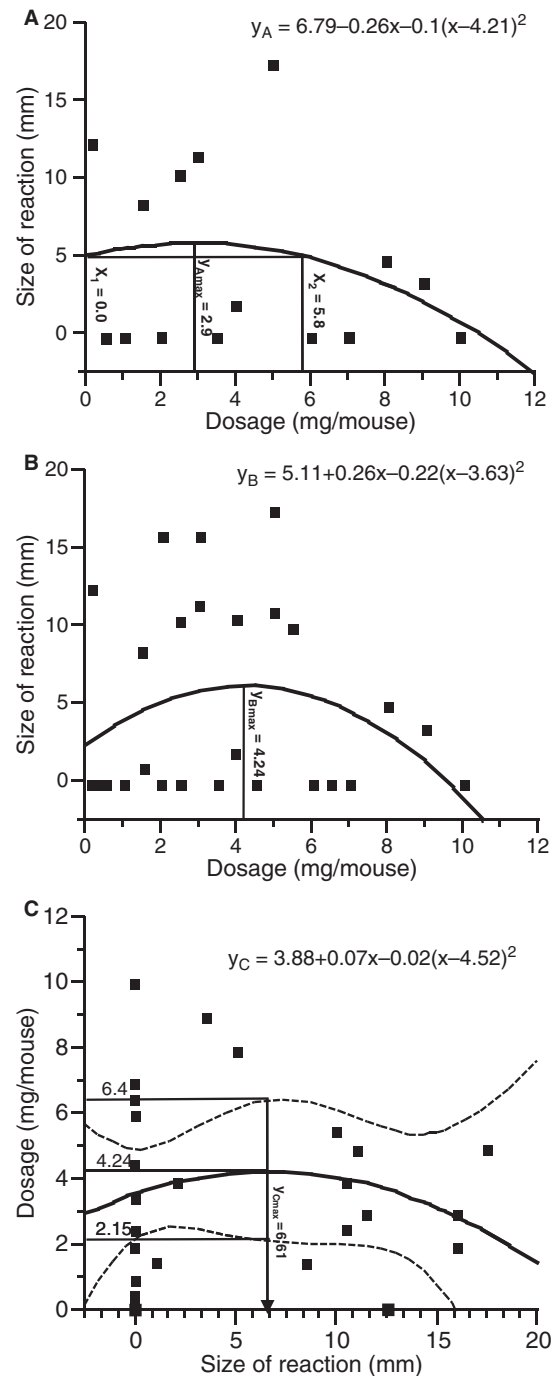


Figure 2 Two-compartment surface response design. Each square represents the passive cutaneous anaphylaxis (PCA) result of one mouse in the dose-response experiment. Fifteen mice were used in each of the two compartments. The polynomial line (y_A , y_B and y_C) in each graph is determined by all the coordinates of the plot. (A) Results from the first compartment ($n = 15$). The doses of compartment two were concentrated around the intersections between y_A and the cut-off line $y = 5$ (x_1 and x_2) and the y_{Amax} . (B) Results from both compartments ($n = 30$). The dosage giving the largest PCA reaction is $y_{Bmax} = 4.24$ mg/mouse. (C) Approximation of the 95% confidence interval of the optimal dose found by exchanging the y -axis with the x -axis from plot B and creating a new polynomial line with its confidence interval (thin lines). The approximated confidence interval for the dose 4.24 mg/mouse is $<2.15-6.4>$.

nized mice (i.p. challenged group) and four control mice (two sham and two naïve) were challenged with an i.p. injection of 5.0 mg fenugreek. The i.p. challenge was used as a positive anaphylactic control. The mice were exsanguinated immediately after the assessment of the anaphylactic reactions.

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.* [20] was applied: 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 clinical anaphylactic reactions were analysed by contingency table analysis (Fisher's exact test) using Statistical Package for Social Sciences (SPSS version 14.0; SPSS Inc., Chicago, IL, USA).

Serum mouse mast cell protease-1 assay. Serum levels of mouse mast cell protease-1 (MMCP-1) were determined at exsanguination (either at day 27 or at day 35) in the acute anaphylaxis experiment with an enzyme-linked immunosorbent assay (ELISA) kit (Moredun Scientific Ltd., Penicuik, Scotland, UK) and performed according to the manufacturer's instructions. For statistical analysis, the 1:100 serum dilutions were used. Results were analysed using one-way analysis of variance on ranks.

Total and fenugreek-specific IgE analyses. Individual sera were analysed on days 1, 13, 27, 34 and 35 for total IgE in the acute anaphylaxis experiment. The levels of total IgE in serum were determined by sandwich ELISA according to the protocol provided by the manufacturer. Mouse IgE ELISA Sets (BD Biosciences, San Diego, CA, USA) were used. Two-way repeated measures ANOVA was used to compare groups, and the results are presented as mean $\pm$ SEM.

Fenugreek-specific IgE antibodies were determined in individual sera at exsanguination in both experiments by the heterologous PCA test [27–30] as described previously [22]. Prechallenge sera were not used for PCA because of the large volume of serum needed to perform the test. Briefly, mouse serum was injected intradermally in rats. Twenty-four hours later, a saline solution containing fenugreek and Evans Blue (Sigma-Aldrich, St. Louis, MO, USA) was administered intravenously (i.v.). After 1 h, the rats were killed and the reactions were read as size in diameter of the blue dot in the skin. All serum samples were diluted 1:4. Results were analysed using one-way analysis of variance on ranks. To adjust for ties in mice with negative results, a normally distributed random number with a mean of 0.1 and SD of 0.25 was added to all these values.

IgG1 ELISA. Polystyrene microtiter plates (Maxisorp, Nunc-Immuno Plate; VWR International, West Chester,

PA, USA) were coated with 0.2 μ g/ml fenugreek extract and incubated for 2 h at 37 °C and then at 4 °C overnight. Serum samples and antibodies were diluted in PBS with 0.05% Tween 20 (PBS-Tw). PBS-Tw was also used as washing buffer between each step. Serum samples were added in duplicates diluted 1:400 and incubated for 2 h at 37 °C. Antibodies were detected by adding 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 (OPD); Sigma-Aldrich]. Absorbance was determined with an ELISA reader (EL808; BioTek Instruments, Winooski, VT, USA) at 450 nm. Detection limit was determined as the mean absorbance for the negative control serum + 3 standard deviations. The results were analysed using one-way analysis of variance on ranks.

IgG2a ELISA. Polystyrene microtiter plates (Maxisorp) were coated with 1.0 μ g/ml fenugreek extract and incubated for 1 h at RT and then at 4 °C overnight. Tris-HCl buffer containing Tween 20 was used as washing buffer between each step. Non-specific binding sites were blocked with 3% skimmed milk in PBS-Tw for 1 h (RT). The IgG2a standard, positive and negative controls and the test sera were applied in duplicates and incubated for 1 h (RT) and then at 4 °C overnight. The standard was diluted by a factor of 4 from 1:6000. Test sera were diluted 1:500. Incubation with biotin-labelled rat anti-mouse IgG2a antibody (clone R19-15; BD Biosciences) for 1 h (RT) followed. Antibodies were detected by incubation with Poly-HRP-streptavidin (Thermo Fisher Scientific Inc, Diagen AS, Rygge, Norway) for 1 h (RT) and developed using TMB solution (Biosource, Invitrogen, Carlsbad, CA, USA). The reaction was stopped using 2N H₂SO₄ and read at 450 nm in an MRX microtiter plate reader (Dynatech Laboratories Inc, Chantilly, VA, USA). Antibody concentrations were given in arbitrary units (AU) per ml.

To prepare the IgG2a standard, Balb/c mice were immunized subcutaneously (s.c.) with 100 μ g fenugreek extract with CpG-ODN 1826 (sequence: 5'TCCATGACGTTCTCCTGACGTT-3'). Pooled serum from normal mice was used as a negative control, while the positive control was serum from fenugreek/CpG immunized mice. AU/ml was determined from the dilution of the standard serum that had an OD value of 0.3 (1:1 536,000) giving a lower detection limit of 1.2×10^4 AU/ml and an upper detection limit of 1.97×10^{11} AU/ml. IgG2a was analysed by one-way ANOVA on log-transformed data.

Splenocyte preparation. Spleen cells from the mice in the acute anaphylaxis experiment were isolated by filtering the spleens through a 70- μ m 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 fenugreek extract at 37 °C and 5% CO₂ for 5 days, the supernatants were collected and stored at -80 °C awaiting analyses.

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 from all groups (challenged, immunized and controls) of the acute anaphylaxis experiment were determined by sandwich ELISA according to the 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. For the IL-5 data, as the response was limited to one group only, statistical testing was not performed.

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 ovalbumin grade VII (OVA; 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 2-(N-morpholino) ethane sulphonic acid SDS running buffer using NuPage 4–12% Bis-Tris gel and SeeBlue Plus2[®] prestained reference standard. The gels were either stained with Simply Blue[™] Safe Stain or electrophoretically transferred onto nitrocellulose membrane (pore size 0.45 µm) in an XCell Blot Module at 30 V and 170 mA for 1 h.

Tris-buffered saline (TBS) containing Tween 20 was used as washing buffer, 5% skimmed milk in TBS as blocking buffer and 1% skimmed milk in TBS as assay buffer. After blocking for 1 h, blots were incubated under gentle shaking overnight at 4 °C with mouse sera diluted 1:100. All further steps were carried out at RT. The blots were washed and incubated successively with two antibodies, first for 1 h with rat anti-mouse IgE (1:1000; Experimental Immunology Unit, University of Louvain, Belgium), and after an intermediate wash, for 1 h with HRP-conjugated goat anti-rat IgG (1:10 000). TMB substrate solution was used for developing.

Immunoblotting was only performed with sera from a selection of mice. Two sera were from immunized mice and three were from challenged mice with an anaphylactic score of 3. All five sera had high total and fenugreek-specific IgE. Control sera were from a naïve mouse with no detectable IgE and from OVA-immunized mice with a high OVA-specific IgE [31]. A control without serum was also included. For the inhibition immunoblotting, serum was preincubated with 2 mg/ml fenugreek extract for 2 h at RT under gentle shaking.

Statistical analysis. Statistical analyses were performed using SigmaStat[®] Statistical Analysis System for Win-

dows Version 3.5 (Systat Software Inc, Point Richmond, CA, USA) unless otherwise stated. All tests were performed two tailed and differences were considered significant if the *P*-values were found ≤0.05. If possible, data were analysed using a parametric Anova test, but if the data could not be transformed to a normal distribution, the non-parametric equivalent, the Kruskal–Wallis analysis of variance on ranks, was used. The *post hoc* tests used for pairwise comparisons were Holm–Sidak and Dunn's, respectively.

Results

An optimal dose of fenugreek was determined using a two-compartment response surface design

The results from the first part of the dose–response experiment (see the Materials and methods section) were plotted in a graph, and the polynomial line $y_A = 6.79 - 0.26x - 0.1(x - 4.21)^2$ was determined (Fig. 2A). From the two lines y_A and $y = 5$, three focus points were determined, namely the intersections between the lines ($x_1 = 0.0$ mg and $x_2 = 5.8$ mg) and the maximum of the polynomial line ($y_{Amax} = 2.9$ mg). In Fig. 2B, results from both compartments were combined. PCA reactions varied from 0 up to 17.5 mm, with 18 of 30 reactions negative (<3 mm). These were distributed throughout the whole range of doses. The polynomial line $y_B = 5.11 + 0.26x - 0.22(x - 3.63)^2$ had its maximum at $y = 4.24$. Thus, the optimal dose of fenugreek extract for inducing fenugreek-specific IgE was determined to be 4.24 mg. Figure 2C shows the approximation of the 95% confidence interval of the optimal dose. The optimal dose of 4.24 mg per mouse was found at the maximum of the line $y_C = 3.88 + 0.07x - 0.02(x - 4.52)^2$, and the confidence interval of this point was <2.15–6.4>.

Clinical anaphylactic reactions were observed after challenge, with MMCP-1 indicating an intestinal origin

Examples of some of the anaphylactic reactions are given in Fig. 3A (2–6). Puffiness around the eyes could be seen as a pale circle, and puffiness around the snout often marked the snout area clearly (Fig. 3A-2 and 3, arrows). Scratching with the hind leg around the head (Fig. 3A-4, arrow) was easily observed. Some scratching is part of normal murine behaviour, and consequently, the behaviour had to be performed with a noticeably increased frequency to be recorded. With stronger reactions it was evident that the mice were uncomfortable and arched backs with piloerection were a clear sign of this (Fig. 3A-5, arrow). The strongest reactions (score 4) were seen in i.p. challenged mice (Fig. 3A-6). These mice would hardly move, even when prodded gently, and they were obviously uncomfortable. Because of ethical consid-

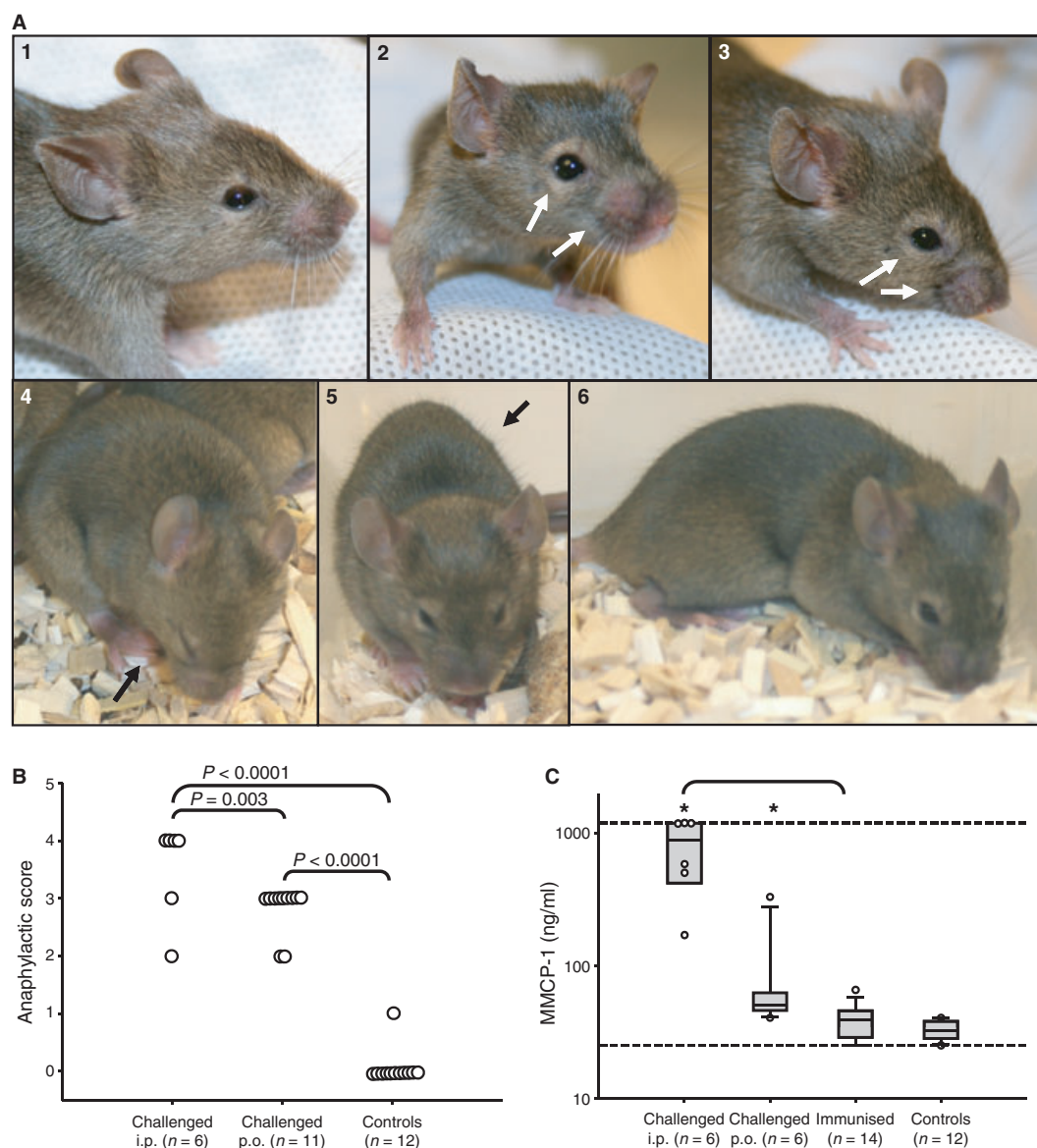


Figure 3 Anaphylactic reactions. (A) Examples of clinical anaphylactic reactions. (1) Normal mouse; (2) and (3) Score 2 – Puffiness around eyes and mouth (arrows; note the circle around the eye); (4) Score 1 – Scratching (arrow) of the nose and head; (5) Score 2 – Arched back with piloerect hair (arrow) and (6) Score 4 – No reaction after prodding. (B) Evaluation of the clinical anaphylactic reactions after challenge with a high dose of fenugreek extract. Results from all challenged mice in the acute anaphylaxis experiment are shown ($N = 29$). Each circle represents one animal. Brackets indicate statistical significant differences (Fisher's exact test, unadjusted values). Controls are a combination of sham-immunized and naïve mice. (C) Mouse mast cell protease-1 levels in serum from all mice in the acute anaphylaxis experiment ($N = 43$). The box plots show median, 25th to 75th percentile, 10th–90th percentile and outliers. In the i.p. challenged group, all individual values are shown (circles). Overall P -value of the Kruskal–Wallis analysis was <0.001 . *Significant difference to controls. Bracket shows significant difference between other groups, as determined by the *post hoc* tests (Dunn's Method, $P < 0.05$). Dashed lines indicate lower (25 ng/ml) and upper (1200 ng/ml) detection limits. Controls are sham-immunized and naïve mice combined.

erations, they were exsanguinated as soon as possible. With a severe fall in blood pressure, it was hard to draw any blood, and these animals were therefore excluded from most of the serum analyses. No mice died after challenge, but this may have been a consequence of the early exsanguination of mice with strong reactions. All mice that had been previously immunized showed reactions with a score of 2 or higher after challenge (Fig. 3B), while the controls

hardly showed any reactions. Both i.p. and p.o. challenged mice showed significantly higher scores than control mice, and i.p. challenged mice had significantly higher scores than mice challenged perorally, as expected.

Mouse mast cell protease-1 in serum was measured as an indication of intestinal anaphylaxis. The results correlated well with the clinical anaphylactic reactions (Fig. 3C). The i.p. challenged group had significantly

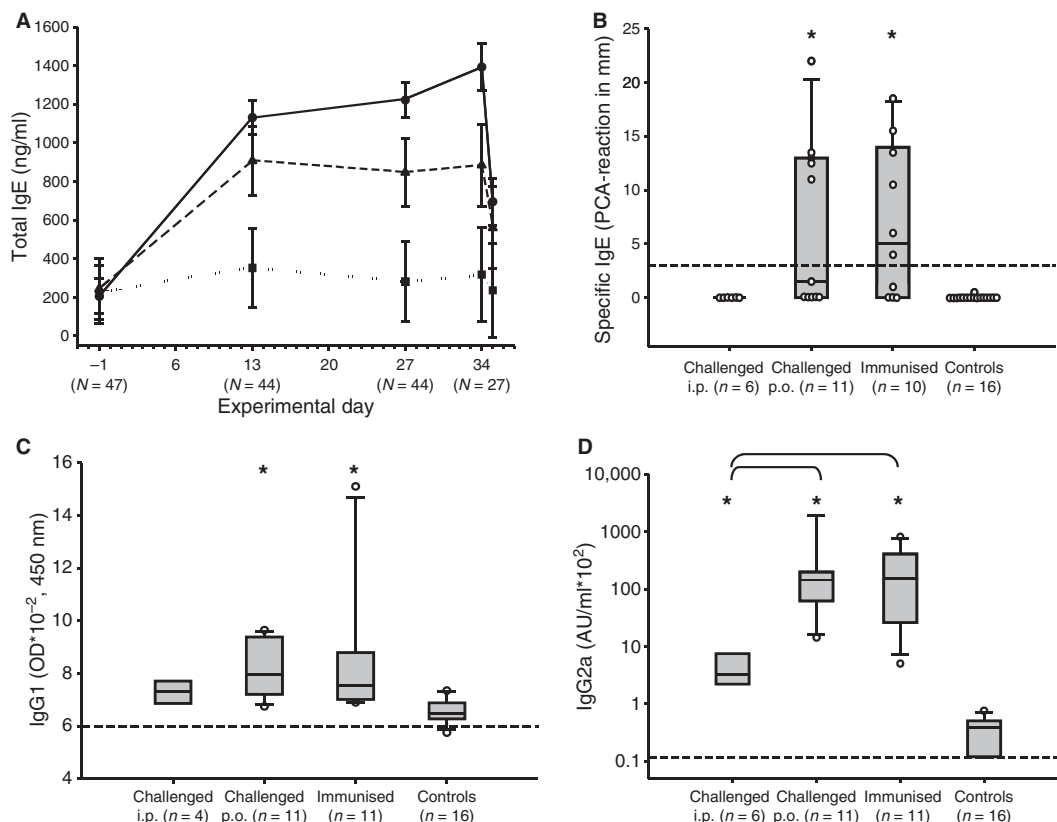


Figure 4 Antibody responses. (A) The development of total IgE during the acute anaphylaxis experiment. The solid line with circles (—●—) represents mice immunized with fenugreek, the dashed line with triangles (---▲---) represents sham-immunized mice and the dotted line with squares (---■---) represents naïve mice. N denoted in the figure indicates the total number of mice at the specific day of treatment. Results are presented as mean $\pm$ SEM. (B) Fenugreek-specific IgE at exsanguination measured by passive cutaneous anaphylaxis from all mice in the acute anaphylaxis experiment ($N = 43$). Overall P -value of the Kruskal–Wallis analysis was <0.001 . (C) Fenugreek-specific IgG1 at exsanguination from all mice in the acute anaphylaxis experiment ($N = 42$) measured by enzyme-linked immunosorbent assay (ELISA). Overall P -value of the Kruskal–Wallis analysis was <0.001 . (D) Fenugreek-specific IgG2a at exsanguination from all mice in the acute anaphylaxis experiment ($N = 42$) measured by ELISA. Overall P -value of the one-way ANOVA was <0.001 . (B–D) The box plots show median, 25th to 75th percentile and 10th–90th percentile. In plot B, individual values (circles) are shown. Controls are a combination of mice treated with CT only and naïve mice. *Significant difference to controls. Brackets show significant differences between other groups as determined by the *post hoc* tests ($P < 0.05$). Dashed lines indicate the lower detection limits.

higher levels of MMCP-1 than both the control groups (sham and naïve) and the immunized mice. The p.o. challenged group showed significantly higher levels of MMCP-1 than the control groups.

There was a strong correlation between the anaphylaxis score and MMCP-1 with a Spearman's ρ rank correlation coefficient of 0.862, $P \leq 0.001$.

Immunized mice had elevated levels of fenugreek-specific and total IgE

To monitor the development of IgE antibodies during the acute anaphylaxis experiment, individual sera were analysed for total IgE (Fig. 4A). Both in mice immunized with fenugreek (immunized and challenged groups combined) and in the sham-immunized control group, an increase in total IgE was observed. The rise from day -1 to day 34 was significantly higher in both immunized mice and sham-immu-

nized mice compared with naïve mice ($P < 0.001$). On day 35 (after challenge), there was a significant decline of total IgE in the challenged mice ($P = 0.006$), while the small decline observed in the other groups was not significant.

Fenugreek-specific IgE in serum was measured by PCA (Fig. 4B). In the immunized group, 6 of 10 mice had positive PCA reactions. This was statistically significant compared with the control group. Five of 11 mice in the p.o. challenged group showed positive reactions, while in the i.p. challenged group, all six mice had negative PCA reactions. No PCA reactions were observed in any of the control mice.

IgG responses were elevated in all immunized animals

Fenugreek-specific IgG1 and IgG2a antibodies were measured in serum by ELISA (Fig. 4C,D, respectively). IgG1 is a Th2-dependent antibody that in mice has been

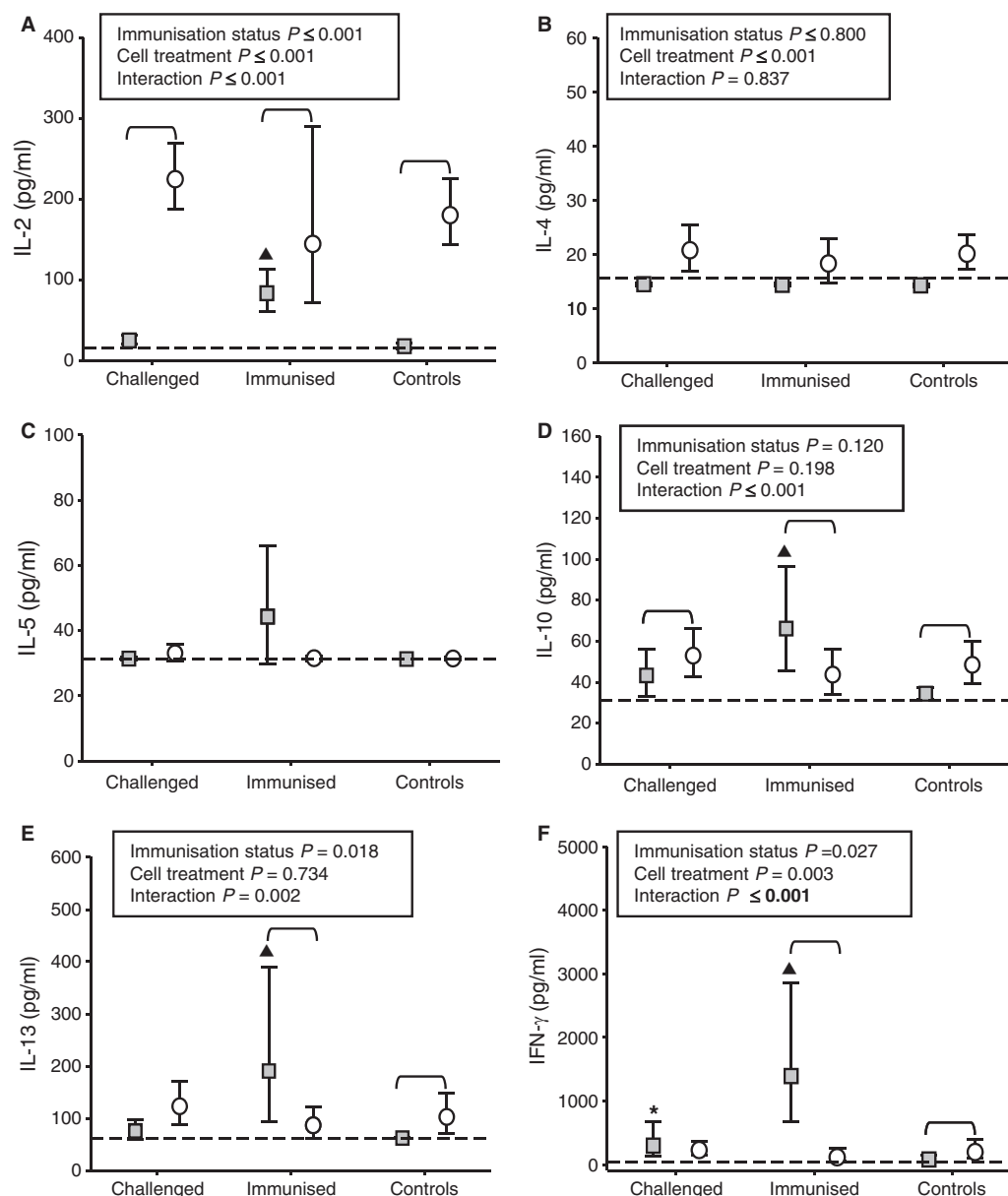


Figure 5 Assessment of cytokine secretion. (A) IL-2; (B) IL-4; (C) IL-5; (D) IL-10; (E) IL-13; (F) IFN- γ . Cytokines were measured in supernatants from spleen cell cultures of all mice in the acute anaphylaxis experiment ($N = 44$). There were 17 mice in the challenged group (both p.o. and i.p. challenges), 11 mice in the immunized only group and 16 control mice. Cells from each spleen were incubated with fenugreek extract (grey boxes) and in plain medium (white circles). Results were analysed using two-way repeated measures ANOVA on log-transformed data and 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 (Holm-Sidak method, $P < 0.05$). $\blacktriangle$ Significantly higher than the other groups within fenugreek-stimulated cells. *Significantly higher than controls within fenugreek-stimulated cells. Dashed lines indicate the detection limits of the assays. Note the different scales.

shown to contribute to anaphylactic reactions, while IgG2a is a measure of the Th1 component of the response. Immunized and p.o. challenged mice had significantly elevated IgG1 and IgG2a levels compared with controls. The i.p. challenged group, however, showed slightly lower levels, and in contrast to IgG2a, the levels of IgG1 in this group were not significantly different from the controls.

Despite a general inhibitory effect of fenugreek, Th2 cytokine concentrations were increased *ex vivo* after exposure

Cytokines were measured in supernatants of spleen cells stimulated *ex vivo* with either fenugreek extract (stimulated) or plain culture medium (unstimulated). The results were analysed with regard to cell treatment (fenugreek versus medium) and immunization status

(challenged, immunized and controls) as shown in Fig. 5.

With regard to the immunization status, there were no differences between the groups of mice in cytokine release from unstimulated cells. In general, the fenugreek extract had an inhibitory effect on cytokine release compared with medium. This was observed in both controls and challenged mice for all cytokines and for IL-2 and IL-4 in all groups [$P(\text{cell treatment}) \leq 0.001$; Fig. 5A,B]. A significant interaction between cell treatment and immunization status [$P(\text{interaction}) \leq 0.001$] for IL-10, IL-13 and IFN- γ (Fig. 5D–F) shows that the effect of cell treatment (exposure or not) was dependent of immunization status. After fenugreek stimulation, IL-10, IL-13 and IFN- γ levels were higher with spleen cells from immunized mice than from challenged mice and from control mice. This could also be seen for IL-5 (Fig. 5C). The pattern was similar for IL-2, but the levels of IL-2 from fenugreek-stimulated cells did not exceed the IL-2 levels of unstimulated cells for any of the groups (Fig. 5A).

In a separate experiment, we examined the survival of spleen cells after 54- and 120-h incubation with or without fenugreek extract using Trypan blue exclusion. Cells were counted manually in a Bürker chamber. The viability after 54 h was found to be ~80% and after 120 h ~90% for both unstimulated and fenugreek-stimulated cells.

Antibody-binding patterns resemble those seen in human patients

SDS-PAGE and Western blotting were used to examine the pattern of protein bands in the fenugreek extract and to determine the IgE antibody-binding patterns in sera from fenugreek-immunized mice. The pattern of fenugreek protein bands separated on the SDS-PAGE gel showed a characteristic quintet of proteins at 50, 55, 58, 62 and 74 kDa (Fig. 6A). The selected sera from the fenugreek-immunized mice used in Western blotting had both high total and fenugreek-specific IgE. Three of the mice had an anaphylactic score of 3 after challenge, while the other two had not been challenged. Immunoblotting showed binding to several bands between 50 and 100 kDa (Fig. 6B). The binding patterns revealed individual variability between the mice independent of challenge status. One band at approximately 50 kDa was prominent in all blots as well as a weaker band at approximately 62 kDa (Fig. 6B-arrows). In the two mice with strongest binding, several bands were prominent (Fig. 6B-lanes 1 and 3). Preincubation of sera with fenugreek extract totally inhibited binding to all bands (Fig. 6B-lane 2 and 4). No binding to peanut, soy, lupin or OVA was observed (data not shown), demonstrating the specificity of the IgE antibodies.

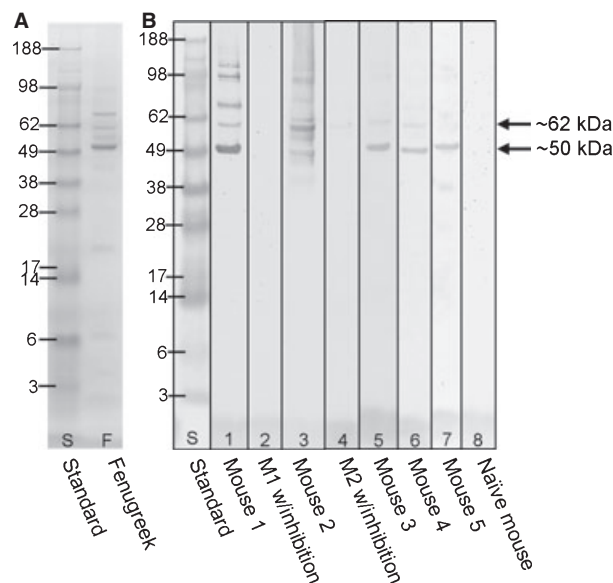


Figure 6 SDS-PAGE and Western blotting. Protein bands of fenugreek extract and antibody-binding patterns were examined using SDS-PAGE and Western blotting. (A) SDS-PAGE of fenugreek stained with Simply Blue™ Safe Stain. Lane S – Molecular weight ladder (SeeBlue® Plus2 Pre-Stained Standard); Lane F – fenugreek. (B) Immunoblot using sera from fenugreek-immunized mice with and without inhibition. Lane S – Molecular weight ladder (SeeBlue® Plus2 Pre-Stained Standard); Lanes 1, 5 and 6 – Challenged mice that displayed strong anaphylaxis (Mouse 1, 3 and 4, respectively); Lane 2 – Same serum as in lane 1 (Mouse 1), but with inhibition; Lane 3 and 7 – Immunized mice, not challenged (Mouse 2 and 5, respectively); Lane 4 – Same serum as in lane 3 (Mouse 2), but with inhibition; Lane 8 – Naïve mouse. Arrows indicate the two bands visible in all positive blots. Protein sizes (kDa) are indicated on the sides of the gel and blots.

Discussion

Using a statistical method previously published [22], we have developed a peroral mouse model of fenugreek allergy with systemic anaphylaxis as the main outcome.

Fenugreek is a novel food in the Western countries, and its use is expanding because of the popularity of Indian cooking. Reports of fenugreek allergenicity have only been published during the last two decades [10–15]. Most reported cases are examples of cross-reactions with other legumes. The possibility of under-reporting cannot be excluded as fenugreek is a hidden ingredient in processed foods, often covered by the term 'spices'. From 2006 to 2010, the Norwegian Food Allergy Register had received 66 reports (of a total of 365) of severe allergic reactions where the patient was sensitized to fenugreek. In eight of these patients, the fenugreek-specific IgE level was higher than the peanut-specific IgE level, and five patients were not peanut sensitized (unpublished data). This suggests that fenugreek may also be a primary allergen and not only a cross-reacting allergen to peanut [13]. It is therefore important to further investigate fenugreek, both as a primary allergen and a cross-reacting allergen

to peanut and other legumes. Also, the natural history of legume allergies other than to peanut, soy and possibly lupin is less known, and more studies are necessary to elucidate this [32]. An animal model as presented will be valuable in these investigations.

When studying allergic reactions in mice, the choice of mouse strain is important. For the study of clinical reactions of allergy and anaphylaxis, the C3H/HeJ is a suitable strain. It will give a mixed Th1/Th2 response, with regard to both cytokines and antibodies [33–36]. As humans also tend to show a mixed Th1/Th2 response [37], the present mouse model is in this respect expected to be a good model for human fenugreek allergy.

The statistical model used to determine an optimal dose for sensitization resulted in a more accurate dose as compared to a traditional block design. In a block design, one usually decides on a low, medium and high dose. If we used a design with, for example, 1 mg as the low dose, 5 mg as the medium dose and 10 mg as the high dose, we would probably conclude that 5 mg was the most adequate dose. When repeating this in a larger experiment, there might be a risk that some mice would not be properly sensitized as this dose lies in the upper part of the confidence interval. In addition, as we are able to test the whole range of doses at once, the model has a potential for using less animals [22].

Fall in rectal temperature is a well-established method for measuring anaphylaxis in mice. However, we found the scoring system valuable in that it mimics well the symptoms of anaphylaxis as seen in humans. To avoid masking of these reactions by stressful handling of the mice during the observation period, we did not attempt to measure body temperature. No fenugreek-immunized mice experienced weak anaphylactic reactions (score 1) upon challenge, which supports our notion that the dose we found was optimal for sensitization. An i.p. challenged group was included to get a positive anaphylactic control, whereas the perorally challenged group reflects the natural way of exposure to foods. Both groups experienced strong anaphylaxis, reflected both in the clinical scores and in MMCP-1 in serum. Serum MMCP-1 has been showed to be a marker for mucosal mastocytosis and increased gut permeability [38] as well as for mast cell-dependent intestinal inflammation [39]. The MMCP-1 response thus indicated an intestinal origin of the anaphylactic reactions.

Total IgE levels were used to monitor the development of IgE as it has been shown that total and specific IgE are well correlated in mice [40–42]. After challenge, a drop in total IgE was observed in all mice; however, this was only significant in the fenugreek-immunized mice. Sham-immunized mice also showed elevated total IgE. The rise in total IgE in these animals could be due to the fact that CT has immunogenic properties or indicate the presence of IgE to allergens in the feed.

Fenugreek-specific IgE on the day of challenge showed a large number of negative reactions in the group that experienced an anaphylactic reaction, particularly in the i.p. challenged groups. This correlates well with the simultaneous fall in total IgE. False-negative skin prick test after anaphylactic reactions has also been observed in humans [43]. Possibly, most of the biologically active IgE was unavailable for serological detection because of extravasation, binding to the allergen or uptake by cells during the anaphylactic reaction. This emphasizes the importance of clinical outcome as a main endpoint of food allergy models [16].

Two main pathways of anaphylaxis have been characterized in the mouse (reviewed in [44, 45]), IgE-mediated (the classic pathway) and IgG-mediated (the alternative pathway). Both pathways include mast cells, and the IgG pathway also includes macrophages. C3H/HeJ mice have been shown to be strong responders with regard to IgG2a [36], and in our model, both IgG2a and IgG1 were elevated after immunization. Previously regarded to depend upon larger amounts of antigens and antibodies, it has recently been demonstrated that the IgG-mediated pathway can be induced with limited amounts of antibody and antigen, similar to the IgE-mediated pathway, and that the combination of the two pathways results in more severe and longer lasting anaphylaxis [46]. Ishikawa *et al.* [46] also suggested that the two pathways could be elicited simultaneously under conditions of conventional sensitization of mice with antigen. Our data suggest that i.p. challenged mice has an involvement of the IgG pathway as well as the IgE pathway. This might be due to (1) the amount of antigen systemically available, (2) the route of administration or (3) the severity of the clinical response. I.p. challenge appears to be different from the p.o. challenge. One explanation for this could be that the allergen must pass the mucosa during a peroral challenge. A peroral challenge will thus better mimic a human allergic reaction.

In experiments investigating cross-reactivity in legumes, we have observed that the fenugreek extract had an inhibitory effect on cytokine release (Vinje *et al.*, manuscript in preparation). This was most pronounced for IL-2, a Th1 cytokine important for cell growth. As the viability of the spleen cells is high after 54- and 120-h incubation with fenugreek extract, this inhibitory effect cannot be contributed to any cytotoxicity of the extract. The inhibitory effect on cytokines is in accordance with the medical properties of fenugreek [8]. It has been used to reduce fevers [7] and has been shown to possess anti-inflammatory and antineoplastic properties [47], as well as having antioxidant activity [48]. Consequently, even a small rise in the cytokine levels may be of biological significance considering the inhibitory effects of fenugreek. Although statistically insignificant, even the small response seen for IL-5 may be of biological relevance on account of this general inhibitory property.

Allergic reactions are considered to be the result of a polarization towards a Th2 response [49]. It has been shown that the development of a Th1-associated inflammatory response could exacerbate allergic disease and that IFN- γ often is present at sites of allergic inflammation [50]. Analysing the cytokine levels in the present study, the Th1/Th2 response pattern seemed to be mixed, but with a tendency towards a Th2 response. The levels of cytokines IL-5, IL-10, IL-13 and IFN- γ released from cells of immunized mice stimulated with fenugreek were all elevated compared with the levels of unstimulated cells. In contrast, IL-2 levels with fenugreek-stimulated cells never exceeded the levels of unstimulated cells. Cells from the fenugreek-challenged group of mice seemed refractory to stimulation by allergen, possibly because they were affected by the ongoing anaphylactic reaction.

The fenugreek protein band pattern on the SDS-PAGE gel was in accordance with other studies, showing a characteristic quintet of proteins between 50 and 74 kDa [13, 14]. Some of the selected sera showed strong binding to fenugreek proteins, while others showed weaker binding. Thus, there were considerable individual variations among the mice, as observed also for human sera [13–15]. The binding pattern obtained for the mouse sera was similar to that of human sera, and the binding to the most prominent band correlated well with the proposed allergen Tri f1 suggested by Fæste *et al.* [14]. This is a 7S vicilin-like protein homologous to known allergens such as Ara h1 from peanut and beta-conglutinin from lupin.

In summary, the present mouse model shows how fenugreek can act as a primary allergen causing anaphylaxis and that fenugreek allergy mimics human fenugreek allergy [10, 11, 13]. The clinical anaphylactic reactions and serum MMCP-1 levels indicate that the mice experienced a systemic allergic reaction of gastrointestinal origin. With the immunoblot inhibition, we have shown specific IgE binding of sera from fenugreek allergic mice to the proposed allergen Tri f1, a homolog of the peanut allergen Ara h1 [14]. A cross-reaction between these two legumes might therefore be likely and should be taken into account when advising peanut allergic patients. Further studies are needed to establish the prevalence of allergic reactions to fenugreek in peanut allergic individuals as well as in the population in general.

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