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Sensitization with 7S Globulins from Peanut, Hazelnut, Soy or Pea Induces IgE with Different Biological Activities Which Are Modified by Soy Tolerance

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Key Words
7S globulins · Food allergy · Sensitization · Tolerance · Brown Norway rats · EuroPrevall

Abstract
Background: It is not known why some foods sensitizing via the gastrointestinal tract are prevalent allergenic foods and others are not. Eating habits, processing, and the food matrix have been suggested to influence the allergenicity of a given food. Factors related to protein structure, such as stability to digestion, have also been suggested. 7S globulins from peanut, hazelnut, soy, and pea were studied to determine whether related proteins would induce a similar sensitization when removed from their 'normal' matrix. ***Methods:*** Brown Norway rats (soy tolerant or nontolerant) were immunized i.p. 3 times with 100 μg purified peanut, hazelnut, soy, or pea 7S without adjuvant. Sera were analyzed for specific antibodies by different ELISAs (IgG1, IgG2a, and IgE), inhibition ELISA, and rat basophilic leukemia cell assay. ***Results:*** The 4 related 7S globulins induced a response with an almost identical level of specific antibodies, but peanut 7S induced IgE of higher avidity than hazelnut and pea 7S which, again, had a higher avidity than IgE induced by soy 7S. Soy tolerance reduced the functionality of IgE without influencing antibody titers. ***Conclusions:*** Although the 4 7S globulins are structurally related allergens, they induce antibodies with different antigen-binding characteristics. Peanut 7S induces IgE of a higher avidity than hazelnut and pea 7S which, again, has a higher avidity than IgE induced by soy 7S. We also show that soy tolerance influences the function of antibodies to peanut 7S. These findings may help explain how antibodies of different clinical significances can develop in different individuals sensitized to the same allergen.
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Introduction

Little is known about why some foods sensitizing via the gastrointestinal tract are prevalent allergenic foods and others are not. Factors such as eating habits, processing, and the food matrix have been suggested to influence the allergenicity of a given food [1, 2]. Factors related to protein structure, such as stability to digestion, have also been suggested [1]. Most plant food allergens belong to only 4 protein families: (i) the prolamin superfamily (comprising 3 major groups: 2S albumins, lipid transfer proteins, and α-amylase/trypsin inhibitors), (ii) the cupin superfamily (comprising the major groups of storage proteins, 7S and 11S globulins), (iii) homologues of the major

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Table 1. Sequence homology between 7S globulins from peanut, hazelnut, soy, and pea

	Soy 7S[a]	Peanut 7S[b]	Pea 7S[c]	Hazelnut 7S[d]
Soy 7S[a]	70–100%	40–49%	51–56%	31–34%
Peanut 7S[b]	40–49%	94–100%	43–52%	33–34%
Pea 7S[c]	51–56%	43–52%	67–100%	35–36%
Hazelnut 7S[d]	31–34%	33–34%	35–36%	100%

Comparison of amino acid sequences using a BLAST search (www.allergenonline.com). Sequence homology is shown as the identity percentage.

The color combination in the columns (vertically) shows the order of sequence homology of the given allergen in the column compared to the allergens in the rows. Color intensity increases with increasing sequence homology.

[a] Soy 7S (Gly m 5), Swissprot acc. P13916 (α-subunit), P11827 (α'-subunit), and P25974 (β-subunit).

[b] Peanut 7S (Ara h 1), Swissprot acc. P43237 and P43238.

[c] Pea 7S (Pis s 1), Swissprot acc. P13918 (vicilin) and P13915 (convicilin).

[d] Hazelnut 7S (Cor a 11) and TrEMBL acc. Q8S4P9.

birch pollen allergen Bet v 1, and (iv) profilins [3]. Jenkins et al. [3] showed that although approximately 65% of plant allergens fall into only 4 structural families this finding does not merely reflect the relative abundance of gene families in plants, but rather indicates that the potential of a plant protein to become an allergen may be associated with membership to 1 of these 4 families and with whether the protein shares conserved structures with 3 dominating allergen families.

The globulin storage proteins are classified into 2 groups, 7S globulins (vicilins) and 11S globulins (legumins), based on their sedimentation coefficient [2, 4, 5].

7S globulins are particularly abundant in legumes and nuts, comprising up to 70% of the protein content. They are salt-soluble storage proteins that are typically trimeric with a relative molecular mass (M_r) between 150 and 200 kDa with a subunit M_r ranging from about 40 to 80 kDa [2, 4, 5]. 7S globulins belong to the cupin superfamily and, while they lack disulphide bonds, they are stabilized by noncovalent hydrophobic interactions, hydrogen bonds, and van der Waals interactions [2, 4, 5]. The basic structural feature of the cupin superfamily is the presence of a common barrel-like architecture that is also described as a double-stranded β-helix [6]. 7S globulins contain 2 copies of this cupin fold and are found to share sequence motifs. These structural motifs are thought to be highly stable, resisting thermal denaturation and certain types of proteolysis [5]. Thus, for this conserved allergen family in which sensitization via the gastrointestinal tract is thought to occur, the stability factor conferred by this fold may potentiate protein immunogenicity and allergenicity [2, 3, 7].

It is suggested that approximately 35–40% sequence identity between allergens is sufficient for cross-reactive IgE binding [3, 8], but cross-reactivity is rare below 50% identity [9]. In legume-allergic patients clinical cross-reactivity is very rare and does not reflect the extensive immunological cross-reactivity between legume antigens found by in vitro assays [10, 11] (see table 1 for the sequence homology between the 4 7S globulins). Jenkins et al. [3] suggested that the limited cross-reactivity between cupins from very closely related plant species compared to the cross-reactivity between e.g. members of the Bet v 1 family can be explained by differences in surface residues as the cupins may not maintain precisely the same main chain conformations; i.e. the surface seen by an IgE molecule changes much more for the cupin superfamily than for other protein families [3]. However, no data are available on the comparative sensitization potential of 7S from related plant species, i.e. their capacity to induce IgE.

In order to study whether related proteins would induce similar sensitizations when removed from their 'normal' matrix, we selected four 7S globulins from plants that have different roles in food allergy (table 2) [12–20]. The amino acid homology between the four 7S globulins varies from 31 to 56% (table 1).

It is difficult to study the sensitization process in humans exposed to an uncontrolled variety of food and inhalant allergens over time. We therefore find it justified to use an animal model where allergen exposure may be controlled. The animals used were Brown Norway (BN) rats, a high IgE-responder strain [21, 22]. For practical reasons, i.e. the amount of purified allergens needed, the intraperitoneal (i.p.) route was used. Experiments were done without the use of adjuvant to avoid skewing the immune response [23, 24] and to assess the intrinsic sensitization potential of these related proteins. Consequently, the IgE titers were too low to induce clinical reactions upon challenge. The functionality of the induced specific IgE response can therefore only be studied in vitro using sensitized effector cells. This approach also has clear animal welfare advantages.

Two series of experiments were performed. One was carried out in soy-tolerant rats bred and kept for several generations on a feed without tree nuts and leguminosa as ingredients, but contaminated with small amounts of

Table 2. Characteristics of the four 7S globulins and their allergenic food

7S globulin	Storage protein in	Allergy prevalence[a], %	Food allergen	Stable to digestion	Content in whole food (7S/g food)	Processing (westernized diet)
Ara h 1	Peanut	0.4–1.6	Major [15]	no [16, 17]	29–42 mg[d]	Roasted at high temperature
Cor a 11	Hazelnut	0.1–1.7	Minor[b] [18]	no[c]	7–16 mg[d]	Lightly roasted
Gly m 5	Soy	0.03–0.7	Minor [19]	no[c]	96–114 mg[d]	Defatted highly processed protein
Pis s 1	Pea	0.2	Major [20]	no[c]	13–36 mg[d]	Blanched/cooked

[a] Estimates of the prevalence of food allergy in general are imprecise and vary between countries and cultures [12–14].
[b] In general, hazelnut allergy is caused by primary sensitization to Bet v 1. This may explain why the storage globulin Cor a 11 is classified as a minor allergen.
[c] Clare Mills (IFR, Norwich, UK) [pers. commun.].
[d] Calculations are based on data from The official Danish Food Composition Database version 7.01 (http://www.foodcomp.dk/v7) and Neil M. Rigby [pers. commun.].

soy proteins. The other series was conducted in rats that were not tolerant to soy and were bred and kept on a similar diet, but without soy contamination.

We report here how soy tolerance reduces the biological activity of antibodies to the related 7S globulins without affecting the titer. In addition, we describe that the four 7S globulins induce comparable amounts of specific antibodies but that the functionality of these antibodies differs. These results may add new knowledge to our understanding of the variability of IgE reactivity and the limitation of IgE titer as a diagnostic marker in food allergy.

Materials and Methods

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of protein-containing fractions was performed using NuPAGE 4–12% Bis-Tris gels (Invitrogen, Paisley, UK) under reducing and nonreducing conditions with MES running buffer according to the manufacturer's protocol. Mark12 MW Standard (Invitrogen) was used as the molecular mass standard and gels were stained using a Coomassie-based stain (SimplyBlue SafeStain; Invitrogen).

Purification of 7S Globulins

7S globulins were purified from peanut (Ara h 1), hazelnut (Cor a 11), soy (Gly m 5), and pea (Pis s 1). The pooled permeation chromatography fractions of each of the purified 7S globulins were judged to be >99% pure by SDS-PAGE (fig. 1). The protein concentration was determined by amino acid analysis [25].

Peanut 7S was purified from red-shelled peanuts (obtained from local suppliers in the UK) as described by Marsh et al. [26]. Hazelnut 7S was purified from raw hazelnuts (produced in Turkey and purchased from a local supplier in the UK) as described by Rigby et al. [27].

Samples of purified 7S globulins were analyzed for the presence of endotoxins by the Lonza endotoxin testing service (Lonza, Verviers, Belgium). The endotoxin content [endotoxin units (EU)/mg protein] was <5 EU [24, 28].

Isolation of Soy 7S

Soybean 7S was partially purified using the method described by Nagano et al. [29], with minor modifications. Briefly, protein was extracted from defatted, unroasted soybean flour (Sigma Chemical Co., Dorset, UK) in water (1:15 w/v) (pH adjusted to 7.0 with 1.0 M NaOH) and stirred for 1 h before clarification by centrifugation (3,500 *g*, 1 h, 18°C). The supernatant was removed and dry sodium bisulfite added to 0.98 g/l (pH adjusted to 6.4 with 1.0 M HCl) and placed in an ice bath at 4°C overnight. After centrifugation (3,500 *g*, 1 h, 4°C) the supernatant was removed, solid NaCl was added to 0.25 M, the pH was adjusted to 5 with 1.0 M NaOH, and it was allowed to stand for 1 h at 4°C. After centrifugation (3,500 *g*, 1 h, 4°C) the supernatant was removed and diluted with 2 volumes of ice cold water adjusted to pH 4.8 with 1.0 M HCl. The resulting precipitate (crude 7S globulin) was removed by centrifugation and the pellet redissolved in 25 mM sodium phosphate buffer (pH 7.5) and 150 mM NaCl. The pH was maintained at 7.0–7.5 during dissolution by the careful addition of 1.0 M NaOH until the protein had fully dissolved. Residual insoluble protein was removed by centrifugation (30,000 *g*, 30 min, 15°C). The resulting crude 7S globulin was subjected to preparative gel permeation chromatography using a Superdex Preparative Grade S200 column (1.6 × 60 cm; GE Healthcare, Little Chalfont, UK) attached to an AKTA BASIC chromatography system (GE Healthcare) equilibrated in sodium phosphate buffer (150 mM NaCl, pH 7.5). Protein was eluted with the same buffer using a flow rate of 1.0 ml/min and absorbance monitored at 220 and 280 nm. The protein-containing fractions were analyzed by SDS-PAGE. Fractions containing polypeptides of M_r 67, 63, and 48 kDa corresponding to the α, α′, and β subunits of Gly m 5 [30] were pooled, concentrated by ultrafiltration, and stored in aliquots at –20°C until use.

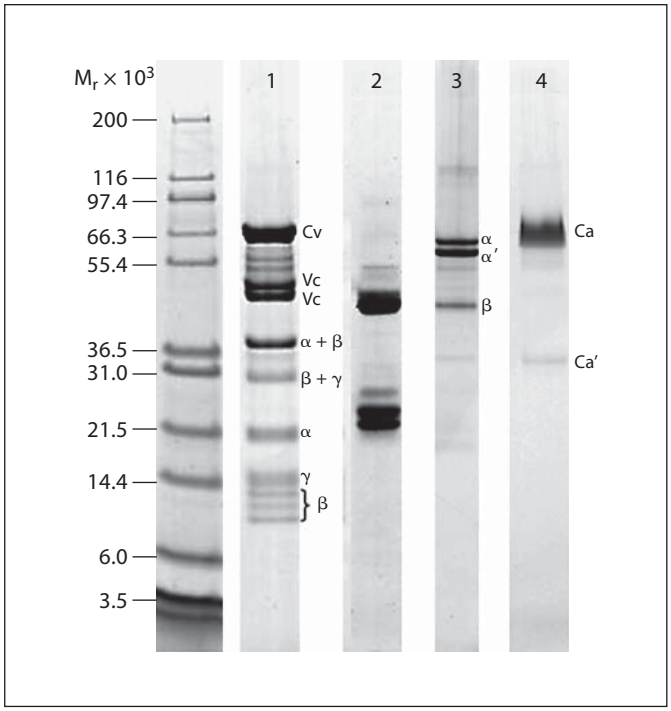

Fig. 1. SDS-PAGE analysis of purified 7S globulins after reduction. Lane 1: pea 7S (Pis s 1) comprising convicillin (Cv), vicilin (Vc) and vicilin subunits γ, β, and α. Lane 2: hazelnut 7S (Cor a 11). Lane 3: soy 7S (Gly m 5) comprising α, α′, and β subunits. Lane 4: peanut 7S (Ara h 1) comprising conarachin (Ca) and small conarachin (Ca′). The blurred appearance of several of the bands is due to glycosylation. SDS-PAGE was undertaken using NuPAGE 4–12% Bis-Tris gel, MES running buffer, and Mark12 MW Standard; staining was performed with Colloidal Coomassie Blue (Invitrogen).

Isolation of Pea 7S

Pea 7S consisting of a mixture of vicilin and convicilin [31] was prepared according to the method described by Suchkov et al. [32]. Defatted pea flour was prepared from ground marrowfat peas (c.v. Princess) by extraction with hexane (twice) at room temperature (RT). The resulting meal was extracted with water (1:5 w/v) (pH adjusted to 8.0 with 1.0 M NaOH) and stirred for 90 min at RT. After centrifugation (3,000 *g*, 35 min, 15°C), the supernatant was collected, solid NaCl was added to 0.5 M, and the pH was adjusted to 4.8 with 0.1 M HCl (containing 0.5 M NaCl). After centrifugation (3,000 *g*, 35 min, 15°C), the supernatant was collected and diluted to 0.3 M NaCl with distilled water. The resulting precipitate was removed by centrifugation (3,000 *g*, 35 min, 15°C) and the supernatant was retained and diluted with 1 volume of distilled water (4°C). The resulting precipitate (crude pea 7S globulin) was recovered by centrifugation (3,000 *g*, 35 min, 2°C). The crude 7S globulin was dissolved in 25 mM sodium phosphate, pH 7.6, containing 0.5 M NaCl and subjected to preparative gel permeation chromatography as described above for soy 7S using 25 mM sodium phosphate buffer, pH 7.6, and 500 mM NaCl. Protein-containing fractions were analyzed by SDS-PAGE and those containing a range of coeluting polypeptides of M_r 10–70 kDa corresponding to the posttranslationally processed pea 7S globulin were pooled, concentrated by ultrafiltration, and stored in aliquots at –20°C until use.

The purity and authenticity of proteins was adjudged using criteria developed for the EuroPrevall allergen library [33]. Thus, the identity of 7S globulins was confirmed by in-gel trypsin digestion followed by Maldi-ToF mass spectrometry as described by Rigby et al. [27] and yielded significant MOWSE scores [34] when the resulting peptide mass profiles were searched against the NCBI nonredundant protein database using the Mascot program from Matrix Science (http://www.matrixscience.com/). Protein purity was adjudged using a combination of SDS-PAGE and gel filtration chromatography, the latter ensuring proteins were in their correct oligomeric form and did not contain either aggregates or breakdown products not discernable using denaturing techniques such as SDS-PAGE.

Animal Diet

To avoid the induction of tolerance against the examined proteins, animal diets were designed to be free of legume proteins and produced at the National Food Institute (DTU, Denmark). The nutrient content was in accordance with rodent diet AIN-93 [35], and all ingredients were ground to a similar particle size to ensure a homogeneous mixture. Two diets without leguminosa were produced, both of which contained potato protein and fish meal as protein sources but differed in whether the main starch source was maize flour (diet A) or rice flour (diet B). Diets were analyzed for soy and peanut proteins using a Peanut Assay Kit (Neogen Europe) and a Soy Residue Kit (Elisa Systems, Windsor, Qld., Australia) in accordance with the manufacturers' instructions.

Animals

The male and female rats, 4–6 weeks old, were from our own colony of inbred high IgE-responder BN rats. Diet and acidified water (pH 3.5) were given ad libitum. Animals were kept in Macrolon cages (2 per cage) at 22 ± 1°C with a relative humidity of 55 ± 5%, air change 10 times/h, and electric light from 9:00 a.m. to 9:00 p.m. The animals were inspected twice daily and body weights were recorded weekly. Upon termination of the experiments all animals were anaesthetized by carbon dioxide inhalation and killed by exsanguination. The animal experiments were carried out at the DTU facilities under conditions approved by the National Agency for Protection of Experimental Animals and by the in-house committee for animal care and use.

Animal Sensitization Studies

To examine the sensitization potential of 7S globulins from peanut, hazelnut, soy, and pea, groups of BN rats were immunized i.p. on days 0, 14, and 28 with 0 (control) or 100 μg of purified peanut, hazelnut, soy, or pea 7S in 0.5 ml PBS (pH 7.2; 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, and 1 mM KH_2PO_4) per rat per immunization. Blood was collected on day 35. The dose of 100 μg purified 7S globulin per rat per immunization was chosen based on previous sensitization studies with peanut 7S globulin [16], milk, and Brazil nut allergens (unpublished data). Sensitization studies were performed in rats bred and kept on diet A for at least 3 generations (soy tolerant) and in rats bred and kept on diet B for 2 (nontolerant) generations. Experiments in soy-tolerant rats were

performed with 12 rats per group (6 rats of each sex), whereas experiments in nontolerant rats were performed with 6 rats per group (3 rats of each sex) because of limited amounts of purified 7S globulins.

For each of the four 7S globulins, positive control sera were produced in soy-tolerant BN rats by i.p. immunization with 0.5 ml of 100 μg 7S/ml per rat adsorbed on 12 mg $Al(OH)_3$ on day 1 and with 0.5 ml of 20 μg 7S/ml per rat on days 21, 35, and 49. Blood was collected at sacrifice (day 56).

In all experiments sera were stored at –20°C until use.

Enzyme-Linked Immunosorbent Assay

The sera from sensitization studies were analyzed for 7S-specific IgG1, IgG2a, and IgE and for total IgE. Known positive and negative serum control pools were included on each plate. Antibody titers were expressed as $\log_2$ titers and calculated using 4-parameter analysis KC4 version 2.7 (Bio-Tek Instruments, Inc., Winooski, Vt., USA). Detection limits were determined as the mean absorbance for the negative control serum + 3 standard deviations (SD).

IgG1 cross-reactivity between the four 7S globulins was examined by measurement of the binding capacity of serum pools from each group by IgG1 inhibition enzyme-linked immunosorbent assays (ELISAs) (fig. 3, 4) and analysis of individual serum samples by IgG1 ELISAs (fig. 6, 7).

For all ELISA experiments, washing procedures and the development of the enzymatic reaction were as described previously [36]. All reagents and incubation periods were at RT for 1 h unless otherwise noted.

ELISAs for the Detection of Specific IgG1 and IgG2a

For the measurement of the specific IgG1 and IgG2a responses against peanut, hazelnut, soy, or pea 7S in serum, plates (96-well, MaxiSorp; Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 μl/well of 0.5 μg of 7S/ml in carbonate buffer (pH 9.6; 15 mM Na_2CO_3, 35 mM $NaHCO_3$). For the detection of peanut 7S-specific IgG1 and IgG2a, plates were blocked at 37°C with 200 μl/well of 1% BSA (IgG1) or 1% rabbit serum (IgG2a) in PBS/Tween buffer (PBS-T; 0.05% Tween 20). A blocking step was not performed for plates used for the detection of specific IgG1 and IgG2a against hazelnut, soy, or pea 7S as optimization procedures showed no effect of blocking on background absorbance values. Subsequently, plates were incubated with serially 2-fold diluted rat sera (starting at 1:8) in PBS-T before incubation with horseradish peroxidase (HRP)-labeled mouse anti-rat IgG1 or IgG2a (1:2,000; Zymed, San Francisco, Calif., USA) in PBS-T followed by development with TMB One (Kem-En-Tec, Copenhagen, Denmark) for 10 min in the dark. Absorbance values for the detection limits were calculated to 0.1 for all IgG1 assays, 0.2 for IgG2a against peanut, soy, and pea 7S, and 0.3 for hazelnut 7S-IgG2a.

Inhibition ELISAs for Examination of the IgG1 Binding Capacity of 7S Globulins

Assay procedures were as described for the measurement of specific IgG1, except that sera were preincubated with inhibitor solutions. Serum pools were diluted to reach an OD between 0.8 and 1.0 and preincubated (1 h at RT) with serial 10-fold dilutions of purified peanut, hazelnut, soy, and pea 7S (0.1–100 μg/ml) before triplicates of serum/inhibitor mix (and sera with no inhibitor as a control) were added to the wells. Analyses were repeated 6 months apart. Results were expressed as B/B_0, where B corresponds to the specific IgG1 binding to immobilized 7S globulin when a known concentration of inhibitor is present and B_0 corresponds to the binding in the absence of inhibitor. For each serum pool, the concentration of inhibitor that inhibits 50% of binding to the coated 7S globulin (IC_{50}) was determined.

Antibody Capture ELISAs for the Detection of Specific IgE

To avoid the interference of the much higher level of IgG than IgE, assays based on a selective IgE capture were established for the detection of 7S-specific IgE responses. Plates were coated overnight at 4°C with 0.5 μg/ml of mouse anti-rat IgE (HPMAB-123 HybriDomus; Cytotech, Hellebaek, Denmark) in carbonate buffer. After blocking of the remaining active sites for 1 h at 37°C with PBS-T containing 1% rat serum from naïve soy-tolerant rats, plates were incubated with serially 2-fold diluted rat sera (starting at 1:8) and then with digoxigenin (DIG)-coupled 7S diluted to 0.2 μg/ml (DIG-peanut 7S, 10:1), 0.8 μg/ml (DIG-hazelnut 7S, 5:1), 0.2 μg/ml (DIG-soy 7S, 5:1), or 0.6 μg/ml (DIG-pea 7S, 5:1) in PBS-T containing 3% rabbit serum. DIG was coupled to each of the 7S globulins using a DIG Protein-Labeling Kit (Roche, Mannheim, Germany) in accordance with the manufacturer's instructions. After washing, plates were incubated with HRP-labeled sheep anti-DIG (1:1,000; Roche) before development for 20 min in the dark. Absorbance values for the detection limits were calculated to 0.1 for peanut 7S-IgE, 0.2 for hazelnut 7S-IgE, 0.15 for soy 7S-IgE, and 0.4 for pea 7S-IgE.

Sandwich ELISA for the Measurement of Total IgE

Total IgE responses were measured for serum pools by coating plates overnight at 4°C with 0.5 μg/ml of mouse anti-rat IgE (HPMAB-123 HybriDomus; Cytotech) in carbonate buffer. After blocking the remaining active sites for 1 h at 37°C with PBS-T, plates were incubated with serially diluted rat sera (starting at 1:20) and rat IgE standard (Rat Myeloma IgE, 02-9788; Zymed). After washing, plates were incubated with HRP-labeled mouse anti-rat IgE (1:2,000; MCA193P; AbD Serotec, Düsseldorf, Germany) before development for 20 min in the dark.

The concentrations of total IgE in serum samples were interpolated from the linear part of the standard curve for purified IgE (3-parameter analysis, KC4 version 2.7). The average concentration of at least 2 serum dilutions was used for the final calculation.

Rat Basophilic Leukemia Assay

To examine whether the 7S-specific IgE responses were biologically functional, sera were analyzed by rat basophilic leukemia (RBL) assay as described by Bøgh et al. [16], with minor modifications. Briefly, RBL-2H3 cells (DSMZ, Braunschweig, Germany) were cultured in 70% Eagle's MEM with Earle's salt/20% RPMI 1640 medium (v:v) supplemented with 5% heat-inactivated fetal bovine serum (v:v), 100 U/100 μg/ml penicillin/streptomycin solution, 2.5 μg/ml amphotericin, and 2 mM L-glutamine. They were harvested in the stationary phase after over-confluence had been reached. Cells were resuspended (1.5×10^6 cells/ml) in assay medium (Eagle's MEM with Earle's salts supplemented with 100 U/100 μg/ml penicillin/streptomycin solution, 2.5 μg/mL amphotericin, and 2 mM L-glutamine) and incubated overnight in flat-bottomed cell culture microtiter plates (Nunc) for attachment (100 μl/well). Attached cells were sensitized pas-

sively with 50 μl/well of rat serum pools (diluted 1:1) and then washed twice before incubation with 100 μl/well of 10-fold diluted allergen solutions (0.1–100 μg 7S/ml) for cross-linking. After incubation, plates were centrifuged and 25 μl supernatant (specific release) was transferred to a microtiter plate (Nunc). β-Hexosaminidase enzymatic activity, a specific marker of RBL cell degranulation, was detected in supernatants by hydrolysis of the substrate *p*-nitrophenyl-N-acetyl β-D-glucosaminide (PNAG; N9376; Sigma) by the addition of 100 μl/well and incubation for 90 min at 37°C. The reaction was terminated by the addition of 100 μl/well of 0.2 M glycine solution, pH 10.7 (G7126; Sigma). β-Hexosaminidase release was quantitatively measured spectrophotometrically at 405 nm with a reference wavelength of 630 nm using a microplate reader (BioTek Instruments, Inc.). The total release from the remaining intact cells was measured for each well by the addition of detergent (75 μl/well of 0.2% Triton® X-100; X100; Sigma) and incubated for 30 min before centrifugation and the transfer of 25 μl supernatant (total release) to a second microtiter plate.

For control of IgE-mediated degranulation (total IgE release), serum-sensitized cells were stimulated with 1.25 μg mouse anti-rat IgE/ml (553914; BD Pharmingen, San Diego, Calif., USA). Negative controls were included on each plate to measure spontaneous release (equivalent to background reading).

For each serum sample, the β-hexosaminidase release was calculated according to the following equation:

$$\%\ \text{Specific release} = \frac{\text{Specific release (stimulated with 7S globulins)}}{\text{Total release (lysed with detergent)}} \times 100\%$$

As the IgE-mediated degranulation (total IgE release) for serum pools was 30–40% for all serum pools and no statistically significant difference was found between groups, results are expressed as the percent of maximum biological release:

$$\beta\text{-hexosaminidase release}\ (\%) = \frac{\%\ \text{Specific release}}{\%\ \text{Total IgE release}} \times 100\%$$

To ensure that the specific degranulation of RBL cells was mediated by cross-linking of IgE and not IgG, cells sensitized with positive and negative control serum pools were stimulated with goat anti-rat IgG (H+L; Zymed) and mouse anti-rat IgG1 and IgG2a (Zymed). The degranulation of RBL cells when stimulated with anti-rat IgG, IgG1, or IgG2a was not different from the observed spontaneous degranulation (data not shown).

Statistical Analysis

All statistical calculations were carried out using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, Calif., USA). ELISA results expressed as antibody titers were examined using nonparametric statistical analysis because the distributions were not normal for all experimental groups. The statistical significance of differences between 2 groups was analyzed using the Mann-Whitney t test. The Kruskal-Wallis test followed by Dunn's multiple comparisons test was used for comparisons of more than 3 groups; RBL results were examined using a 2-way ANOVA. Differences between experimental groups were regarded as significant when $p \leq 0.05$.

Results

Low Soy Content in the Diet Induces Tolerance without Measurable Antibodies

Diet A with maize flour contained low amounts of soy protein. The feed from different batches contained from 1 to 25 μg soy/g diet, corresponding to a daily oral intake of 2–50 μg soy 7S per rat. Peanut protein was below the detection limit (<1 μg peanut/g sample).

As the diet is powdered, animals also inhale small amounts of it [36]. Untreated control animals did not have detectable antibodies to soy 7S (data not shown). The dietary soy exposure resulted in low antibody responses when sensitized to soy 7S. This effect was abrogated when animals were kept on soy-free diet B for 2 generations (fig. 2).

7S Globulins from Peanut, Hazelnut, Soy, and Pea Induce Similar Antibody Titers

Rats were bred and kept on diet B with rice flour free of soy proteins (detection limit <0.125 μg soy/g flour) for 2 generations. Comparing sensitization studies in these nontolerant rats with the result from the soy-tolerant rats showed that soy tolerance did not influence the IgG1 or IgE response to peanut, hazelnut, or pea 7S measured by ELISA (fig. 2). Similar results were found with IgG2a (data not shown). This is in accordance with our experience where IgG1 and IgG2a titers correlate in the BN rat.

Soy Tolerance Decreases the Binding Capacity and Functionality of Antibodies to Other 7S – in Particular to Peanut 7S

In contrast to the similarity in antibody titers shown in figure 2, the inhibition ELISAs with peanut 7S showed a 4.5-times stronger IgG1 binding of serum pools from nontolerant rats compared to soy-tolerant rats. There was no effect for pea and hazelnut 7S (fig. 3, 4). Tolerance also affected IgG1 binding in the soy-immunized rats (1.75 times stronger in the nontolerant rats), but here there was also a difference in titers (fig. 2–4). It should be noted that inhibition curves for IgG1 binding are shown using a logarithmic scale for inhibitor concentration (fig. 4). This masks the difference compared to the IC_{50} values shown in figure 3 using a linear scale.

Soy tolerance also decreased the functionality of specific IgE in the RBL assay. Serum pools from soy-tolerant animals induced a statistically significantly lower degranulation of RBL cells compared to nontolerant rats (fig. 5). Again, this effect was strongest for peanut, less strong for soy and hazelnut, and there was no effect for

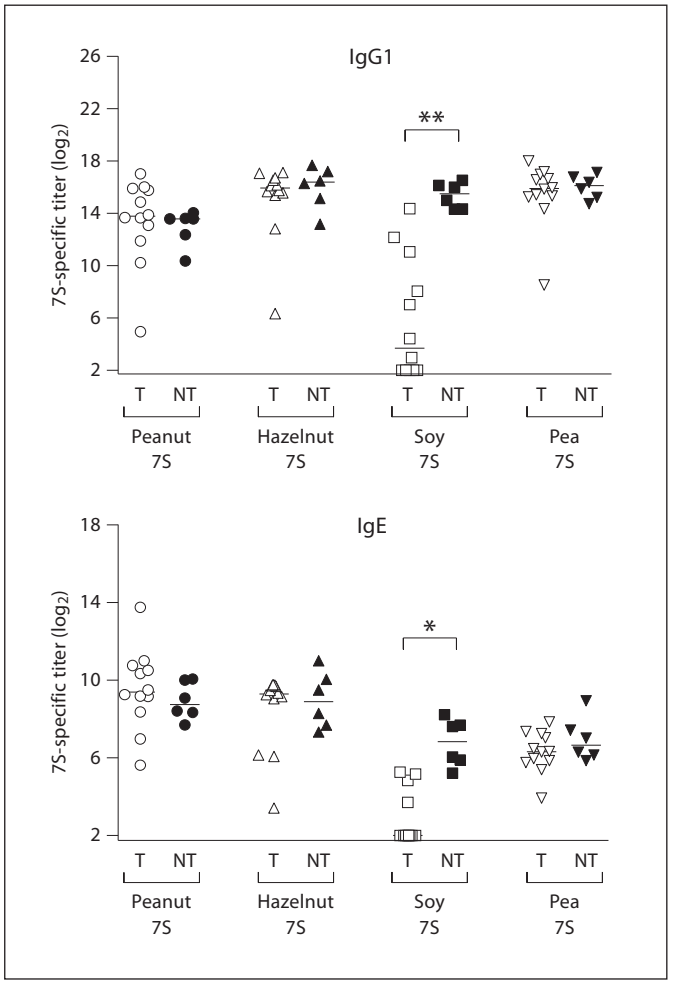

Fig. 2. Specific IgG1 and IgE response in sera from BN rats immunized i.p. with 7S globulins from peanut, hazelnut, soy, or pea. Groups of rats were dosed on days 0, 14, and 28 with 100 μg of peanut 7S (○), hazelnut 7S (△), soy 7S (□), or pea 7S (▽). Sensitization studies with each of the four 7S globulins were conducted in soy-tolerant rats (T; open symbols) bred on a diet containing minor amounts of soy proteins (diet A) and in nontolerant rats (NT; closed symbols) bred on a rice-based leguminosa-free diet (diet B) for 2 generations. Serum samples were obtained at sacrifice (day 35) and analyzed by ELISA. Each symbol represents an animal. Horizontal bars indicate median values. Asterisks over a horizontal line indicate statistically significant differences between the 2 given groups. * $p \leq 0.05$; ** $p \leq 0.01$.

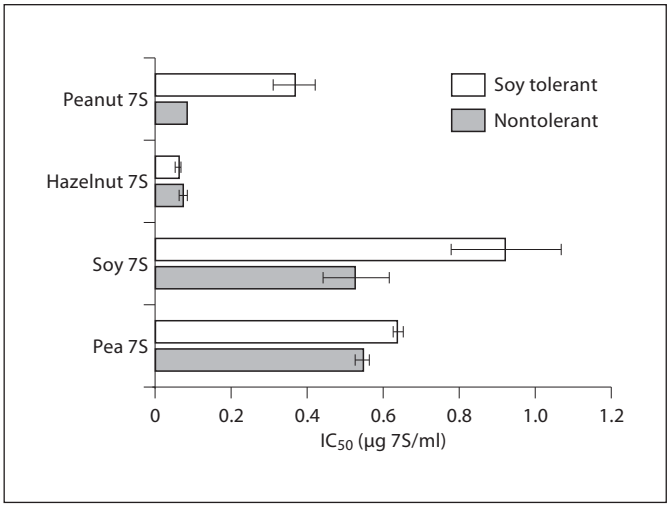

Fig. 3. Comparison of the IgG1 binding capacity of sera from soy-tolerant and nontolerant rats to purified 7S globulins. Serum pools from sensitization studies were preincubated with 10-fold dilutions of peanut, hazelnut, soy, and pea 7S (0.0001–100 μg/ml). Bars represent the mean IC_{50} values for serum pools inhibited with the corresponding 7S globulin. Error bars represent ±SD. Mean values were calculated as the average of 2 measurements (each representing triplicate pipettings of the same serum/inhibitor mix) performed 6 months apart.

pea 7S. The lack of IgE-mediated degranulation by anti-soy sera from tolerant animals may also be a result of low IgE titers.

Soy Tolerance Changes Cross-Reactivity

Soy-tolerant animals immunized with soy 7S developed IgG1 with very limited or no cross-reactivity to hazelnut, peanut, and pea 7S (fig. 6). In the nontolerant rats, immunization with soy 7S induced antibodies which bound with a high affinity to peanut and pea 7S, but no effect was found on hazelnut 7S binding. In contrast, IgG1 from soy-tolerant animals immunized with peanut or hazelnut 7S (fig. 7) readily bound to soy 7S, but this cross-reactivity did not exist with IgG1 from the corresponding nontolerant rats. Again, the effect on pea was minimal as soy tolerance did not seem to influence pea cross-reactivity (fig. 7).

The cross-reactivity was not strong enough to produce an IC_{50} in an inhibition ELISA or cross-link IgE in the RBL assay (data not shown).

In summary, the data shows that soy tolerance has the most pronounced effect on peanut 7S sensitization on IgE and IgG responses as shown in the inhibition ELISA, RBL assay, and cross-reactivity (fig. 3–7). There is a small effect on hazelnut 7S, and the effect on pea 7S sensitization is only marginal (fig. 3–7). Finally, data shows that soy 7S share epitopes with peanut, pea, and hazelnut 7S and that soy tolerance influences antibody formation to these epitopes (fig. 6, 7).

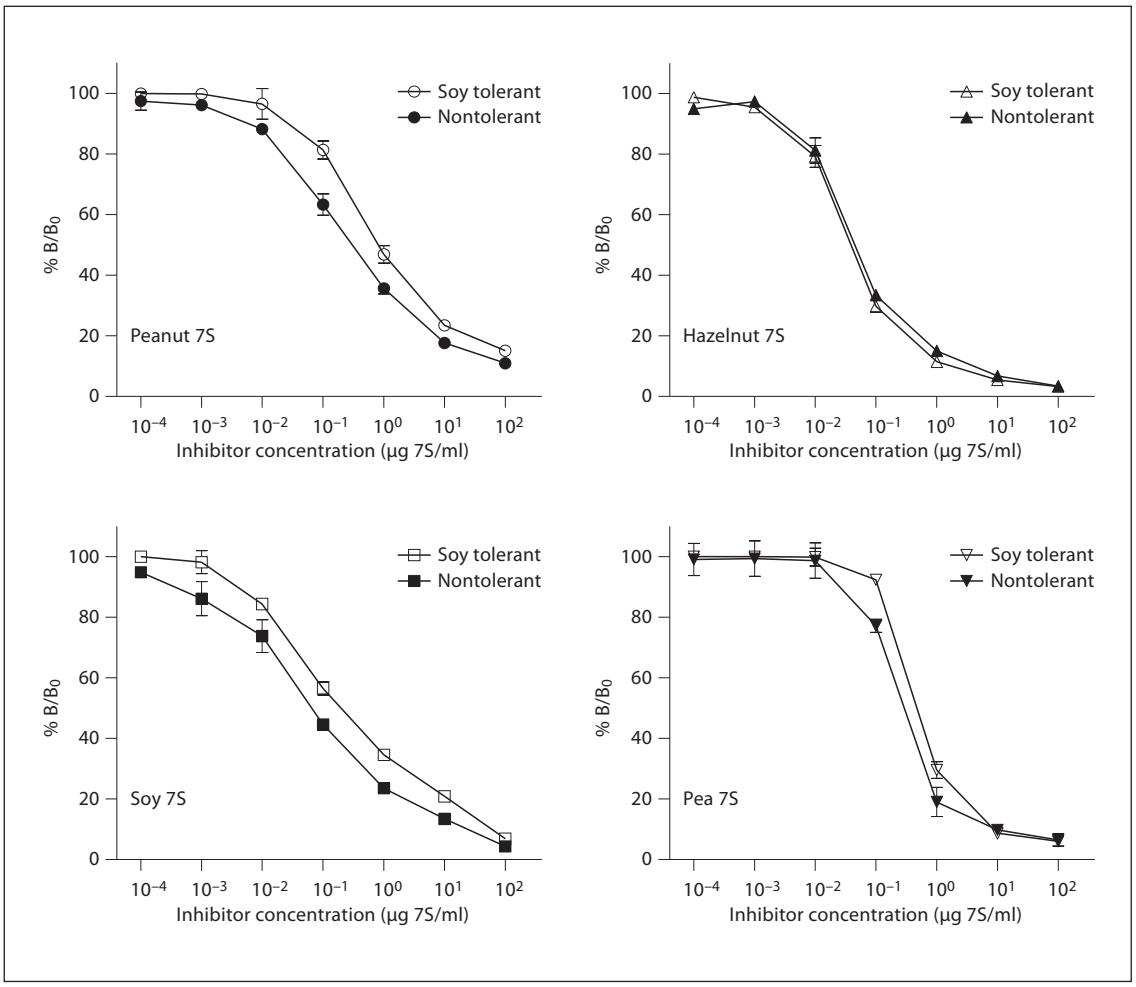

Fig. 4. Inhibition curves for sera from soy-tolerant and nontolerant rats to purified 7S globulins. Serum pools from sensitization studies were preincubated with 10-fold dilutions of the corresponding 7S globulin (peanut, hazelnut, soy, or pea 7S; 0.0001–100 µg/ml). Results are expressed as % B/B_0, where B corresponds to the specific IgG1 binding to immobilized 7S globulin when a known concentration of inhibitor is present and B_0 corresponds to the binding in the absence of inhibitor. Error bars represent ±SD. Mean values were calculated as the average of 2 measurements (each representing triplicate pipettings of the same serum/inhibitor mix) performed 6 months apart.

7S Globulins from Peanut, Hazelnut, Soy, and Pea Induce Antibodies with Different Biological Activity

Although there was a tendency to a lower specific IgE response in the nontolerant animals immunized with soy or pea 7S compared to immunization with peanut and hazelnut, the difference was not statistically significant. The specific IgG1 response to peanut 7S in nontolerant animals was statistically significantly lower than the IgG1 response to the other three 7S globulins (fig. 2; $p \leq 0.05$ compared to soy 7S and $p \leq 0.01$ compared to hazelnut and pea 7S); however, this may be a consequence of the lower sensitivity of the peanut 7S-IgG1 assay rather than a decreased immunogenicity of peanut 7S compared to the other 7S globulins. In contrast, in the inhibition ELISA anti-peanut 7S and anti-hazelnut 7S nontolerant serum pools showed a 6- to 7-times higher IgG1 binding capacity (lower IC_{50}) compared to serum pools from rats immunized with soy or pea 7S.

Moreover, in the RBL assay a statistically significant higher degranulation was achieved with sera from nontolerant animals immunized with peanut 7S compared to the other three 7S, whereas sera from hazelnut and pea induced a statistically significantly higher degranulation than did soy 7S.

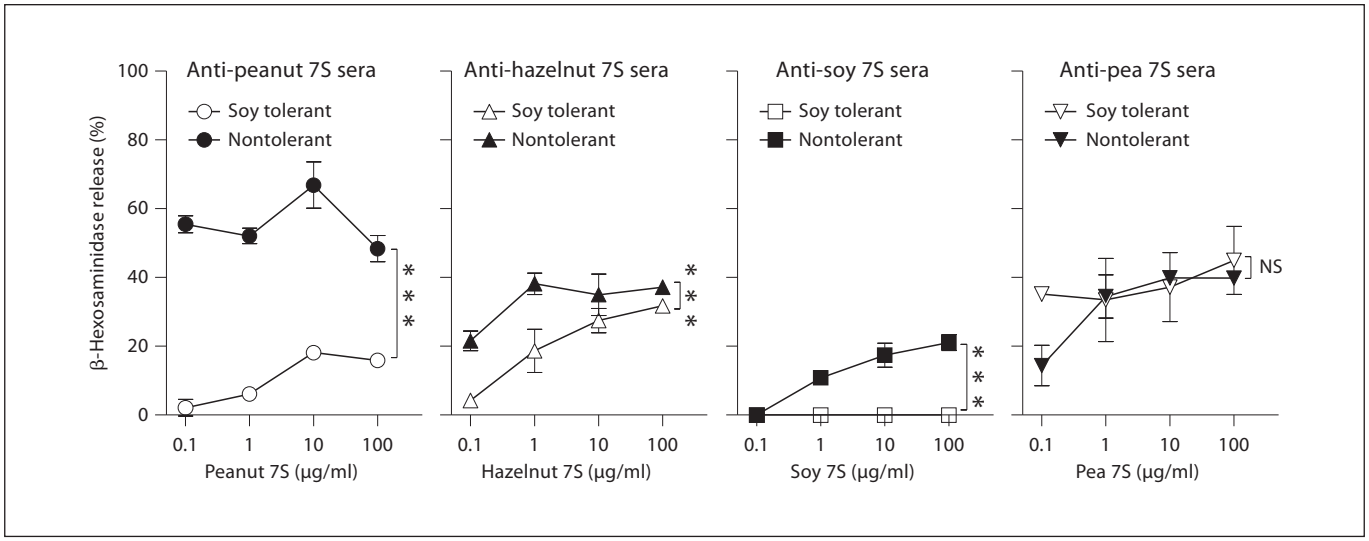

Fig. 5. Allergen-specific degranulation of RBL cells. RBL cells were passively sensitized with serum pools (diluted 1:1) of groups sensitized to purified peanut, hazelnut, soy, or pea 7S. For degranulation, cells were stimulated with dilutions of the corresponding antigen. Sera were obtained from soy-tolerant rats bred on a diet with minor amounts of soy and from nontolerant rats bred on a rice-based diet without leguminosa for 2 generations. Data are presented as the percentage of allergen-specific β-hexosaminidase release in relation to total IgE release. Symbols represent the mean values of duplicates for each serum pool. The allergen-specific release was found to differ statistically significantly ($p \leq 0.001$) between serum pools from nontolerant rats: anti-peanut 7S > anti-hazelnut 7S, anti-pea 7S > anti-soy 7S. Error bars represent ±SD. *** $p \leq 0.001$; NS = Not significant.

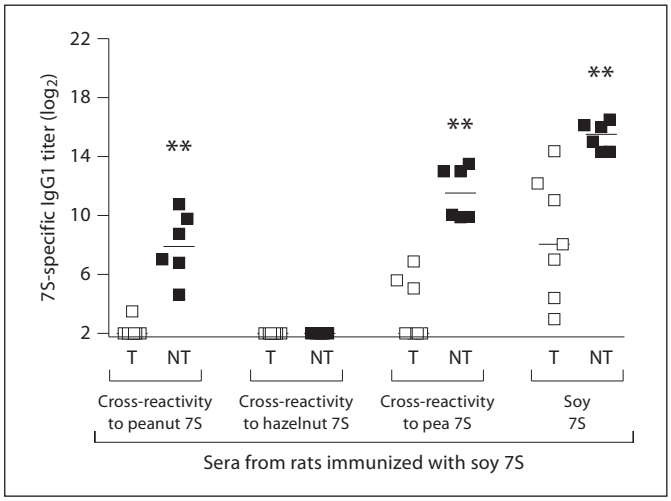

Fig. 6. IgG1 cross-reactivity to peanut, hazelnut, and pea 7S in sera from soy 7S-sensitized rats. Sera from BN rats immunized i.p. with soy 7S were analyzed for specific IgG1 against peanut, hazelnut, and pea 7S by IgG1 ELISA. Cross-reactivity was compared between sera from soy-tolerant rats (T; open symbols) and nontolerant rats (NT; closed symbols). Only sera from rats with an IgG1 response against soy 7S are included. Each symbol represents an animal. Horizontal bars indicate median values. Asterisks indicate a statistically significant difference in a nontolerant group compared to the corresponding soy-tolerant group. ** $p \leq 0.01$.

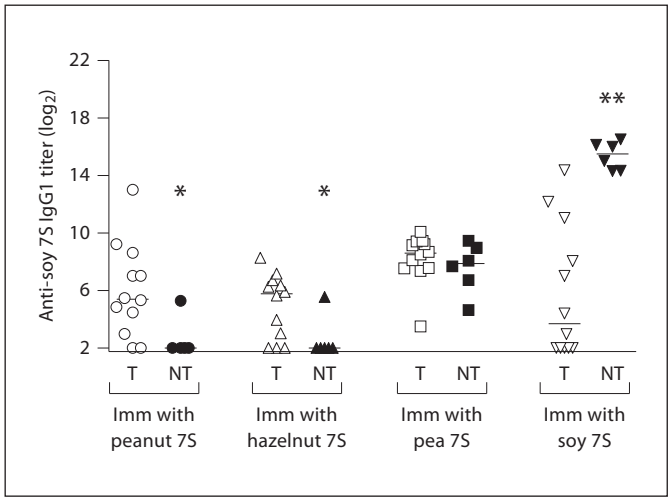

Fig. 7. IgG1 cross-reactivity to soy 7S in sera from rats immunized with peanut, hazelnut, or pea 7S. Sera from BN rats immunized i.p. with peanut, hazelnut, soy, and pea 7S were analyzed for specific IgG1 against soy 7S by IgG1 ELISA. Cross-reactivity was compared between sera from soy-tolerant rats (T; open symbols) and nontolerant rats (NT; closed symbols). Each symbol represents an animal. Horizontal bars indicate median values. Asterisks indicate a statistically significant difference in a nontolerant group compared to the corresponding soy-tolerant group. * $p \leq 0.05$; ** $p \leq 0.01$; Imm = Immunized.

Taken together, these findings show that the four 7S globulins induce an immunogenic and allergenic response in nontolerant rats with an almost identical level of specific antibody but with significant differences in biological activity.

Discussion

There are many unsolved questions concerning the natural history of food allergy. It is known that protein stability may be important for sensitization, but it is still unclear why one food is more allergenic than another. In this study we show that related allergenic proteins having a similar structure and stability may induce IgG and IgE antibodies of different functionality upon sensitization. We also show that tolerance to 1 allergen may affect the functionality of the antibody response to a cross-reacting allergen without affecting the antibody titer.

If rats are fed a diet in which the major components are milk or soy, the rats will have specific IgG to these dietary components [37, 38]. Such animals are unsuited for oral or low-dose i.p. sensitization studies, especially when not using adjuvant. Cross-reacting allergens may not be studied using such animals either [16, 39].

In experimental animal models of mucosal tolerance, tolerance is typically induced by low doses of antigen administered nasally or orally. The lower immune response in tolerant animals compared to controls is demonstrated by subsequent immunization, often with adjuvant [40, 41]. Tolerance may be more readily induced by one antigen compared to another. Tolerance to Bet v 1 was induced by 3 gavage doses of 100 μg [40], whereas it took 50 days of dosing with 100 μg ovomucoid per day in drinking water to induce tolerance [41]. Both experiments were carried out in Balb/c mice. In the present study BN rats were bred on diet A, without leguminosa, based on maize flour contaminated with small amounts of soy for several generations. These animals had no measurable specific antibodies to soy 7S. A comparison of the immunization results with soy 7S in animals bred and raised on diet B (a rice-based diet without soy protein) for 2 generations with the results in rats bred and raised on diet A (containing minor amounts of soy protein) showed that the rats on diet A were tolerant to soy, thereby demonstrating that tolerance was induced in BN rats by a daily oral intake of 2–50 μg soy 7S per rat.

Several mechanisms for the development of mucosal tolerance have been described. These include T cell deletion, T cell anergy, and active suppression by regulatory T cells (reviewed by Horner [42] and van Wijk and Knippels [43]). For oral tolerance it has been shown that high-dose antigen induces T cell deletion and anergy, whereas low-dose antigen induces active suppression by regulatory T cells [40].

Surprisingly, soy tolerance did not seem to affect the sensitization potential of peanut, hazelnut, or pea 7S globulin as specific IgG1, IgG2a, and IgE responses in the tolerant and nontolerant rats were very similar.

When looking at the ability of the peanut, pea, and hazelnut 7S antisera to cross-react with soy 7S, the seemingly identical sera showed a peculiar pattern of reaction. In the soy-tolerant animals immunization with peanut, pea, or hazelnut 7S induced IgG1 antibodies that could bind to soy 7S. The same immunization in nontolerant animals did not show any cross-reactivity to soy.

In our animals mucosal tolerance was induced by small amounts of allergen presumably resulting in active T cell suppression [40]. Tolerance was partly abrogated by i.p. injection of soy 7S but also by i.p. injection of the related 7S globulins, resulting in an antibody response to soy similar to the one induced by soy itself and indicating that tolerance does not exclude the formation of memory B cells. It has been described that memory B cells may be silent, i.e. without a significant differentiation of siblings into antibody-forming cells [44]. Fish et al. [45] present data suggesting that the clonal selection of memory B cells may require a lower affinity immunoglobulin-antigen interaction than does the clonal selection of naïve cells. Due to their low 'affinity cutoff' for stimulation, the participation of memory B cells in immune responses to antigens other than those that initially elicited them may be a rather common occurrence [45].

Our findings may be explained by the presence of memory B cells to soy in the soy-tolerant animals. Antibodies of lower activity/avidity were produced in the tolerant animals when sensitized with the cross-reacting 7S allergens compared to sensitized nontolerant rats. This is in accordance with Fish et al. [45].

In humans with food allergy, specific IgE may be more or less reactive. Serum pools with similar IgE titers from peanut-allergic patients induce more degranulation of mast cells if they recognize many epitopes compared to sera recognizing few epitopes. This finding also correlates with the severity of symptoms [46]. It has been shown that the avidity of IgE and IgG antibodies to Ara h 2 was related to the severity of peanut allergy in peanut-allergic patients [47]. This is in agreement with the finding that the affinity of IgE antibodies to allergens is one of the primary factors influencing the sensitivity of his-

tamine release [48, 49]. Other factors increasing human basophil degranulation are an increased concentration of total IgE and an increased concentration of allergen-specific IgE relative to non-allergen-specific IgE [49]. It has also been shown that antibody avidity is not correlated with antibody titer as it may increase or decrease with increasing titers [50].

Our findings strongly suggest that peanut 7S immunization of soy-tolerant animals results in antibodies of lower avidity compared with the antibodies to peanut 7S produced in nontolerant animals, as illustrated by the results in the inhibition ELISA and the RBL assay. Soy tolerance also seemed to affect IgE avidity to hazelnut 7S, whereas soy tolerance had no effect on pea 7S sensitization measured by inhibition ELISA, RBL assay, or direct cross-reactivity of IgG. That the results of the RBL assay reflect true differences is supported by the fact that the concentration of allergen-specific IgE relative to the total IgE was similar in the compared experiments [49].

The results also show that there is a difference in the allergenicity of the four 7S globulins, which to a certain degree resembles what is seen in humans (in the form of prevalence and severity). What makes peanut 7S more allergenic than pea, hazelnut, and soy 7S? Answering this question is difficult. However, based on this study it seems that something inherent in the allergen structure might be the key to the difference since this study is based on purified proteins where no matrix or processing could influence the outcome and i.p. immunization removed any influence of the gastrointestinal tract.

It has been shown that only small changes in surface amino acid residues may influence sensitizing capacity [51, 52]. A high sequence similarity is thought to increase the chance of shared epitopes of allergens; however, a single amino acid exchange may drastically influence the extent of allergenicity and cross-reactivity [52].

Glycosylation may also influence the immunostimulatory property of a protein [53, 54]. Glycosylated peanut 7S has been shown to act as a Th2 adjuvant by activating dendritic cells to drive Th2 cell maturation. In contrast, deglycosylated peanut 7S did not activate dendritic cells [55]. Whether the glycosylation patterns of the four 7S globulins are sufficiently dissimilar [18, 56] to result in a different sensitizing potential and hence contribute to the differences between the four 7Ss remains to be investigated.

The inhibition ELISA and RBL assay show that although the 4 globulins are related they induce antibodies with a different ability to bind to the antigen. The RBL results indicate that peanut 7S induces IgE of a higher avidity than hazelnut and pea 7S which, again, has a higher avidity than IgE induced by soy 7S.

It may be considered bold to compare inhibition ELISA and RBL results from sensitization studies with different allergens; however, we find it justified when using a controlled system like our animal model and the same assays for all 4 purified 7S globulins.

It is well known in food allergy that the presence and/or level of specific IgE is not sufficient to diagnose food allergy; hence the need for oral provocation [57]. In this study we demonstrated that only measuring antibody titers yields very limited information. The four 7S globulins induced very similar IgG and IgE titers, and only by including results from the inhibition ELISA and RBL assay did the differences in antibody response become apparent. Furthermore, it is important to note that the labeling and coating procedures used for immunochemical assays may result in protein denaturation or the masking of epitopes. It is, therefore, important to carefully consider whether immunochemical and in vitro assays provide information about linear or conformational epitopes and how the assays supplement each other. Such considerations also emphasize the need to utilize well-characterized, authenticated protein preparations for such studies [58].

In the present study, BN rats were sensitized with purified 7S globulins by i.p. immunization without adjuvant, whereas sensitization to food allergens in humans is primarily induced orally or nasally. The i.p. route was chosen mainly because of a limited amount of purified 7S globulins, as approximately 35 mg of purified protein per BN rat is required for oral sensitization when not using adjuvant [59]. Mine and Yang [60] studied how different immunization routes influenced the specificity of IgE response towards ovalbumin and found that the pattern of antibodies to IgE epitopes was dependent on whether mice were immunized i.p. with $Al(OH)_3$, subcutaneously with Freund's complete adjuvant, or orally with cholera toxin. However, regardless of the immunization procedure used, all sera recognized 2 IgE epitope regions which were found to display the highest binding signal intensity [60]. Nonetheless, it is difficult to interpret these results with respect to the influence of the immunization route on antibody specificity and affinity as both the route and adjuvant changed.

It has been shown that mice sensitized to peanut extract can be desensitized with soy extract [61]. Our results show for the first time, that soy tolerance influences the function of antibodies to peanut 7S. This may explain how antibodies of different clinical significance can de-

velop in different individuals sensitized to the same allergen. It also emphasizes the potential complexity of interpreting the influence of dietary patterns in relation to food allergy, e.g. has soy been eaten before peanut or peanut before soy?

We also show that structurally related allergens may induce antibody responses of a different avidity. With further research this finding may contribute to understanding why some food allergies are more common and more severe than others even though they are caused by closely related plants.

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