

ORIGINAL PAPER

Evaluation of allergenicity of genetically modified soybean protein extract in a murine model of oral allergen-specific sensitization

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Clinical and Experimental Allergy

Summary

Background With the development of genetically modified crop plants there has been a growing interest in the approaches available to assess the potential allergenicity of novel gene products. For additional assessment of the potential allergenicity of expressed proteins, informative data can be generated using animal models. Soybean is one of the major source of protein in human and animal nutrition, and has also been well characterized as a major allergenic source. Advances in biotechnology have resulted in an increasing number of genetically engineered foods, and among these soybean is one of the most widespread.

Objective To develop and characterize a murine model of IgE-mediated soybean sensitization induced by intragastric immunization, in the presence of Cholera Toxin, with wild-type soybean extract (wt-SE) or with genetically modified soybean extract (gm-SE).

Methods Balb/c mice born in our animal facilities, from females fed on soy-free food, were fed with the same soy-free food and used in all the experiments. Mice were sensitized by gavages with soybean extracts, and allergen-specific IgE and IgG responses were studied by direct ELISA and ELISA inhibition. Antigen-specific cell proliferation and cytokine production were evaluated in spleen cell cultures.

Results Sensitization with both soybean extracts induced high levels of antigen-specific IgE and IgG1 and low levels of specific IgG2a. Both wt-SE and gm-SE were able to inhibit the binding of specific IgE from mice immunized with gm-SE to the same antigen used for the ELISA coating. A comparable proliferative response was obtained with the homologous as well as with the heterologous extracts.

Conclusion In sensitized mice, we observed a predominantly T-helper type 2 (Th2)-type immune response, with increased soybean-specific IgE and IgG1 antibodies and a concomitant increase of IL-4 and IL-5 production. Results obtained by specific IgE ELISA inhibition and by antigen-specific T cell proliferation demonstrated that wt-SE and gm-SE shared B and T epitopes. The present murine model of soybean sensitization established by the oral route should provide valuable information about risk assessment for food allergy from new proteins of genetically modified foods.

Keywords evaluation of allergenicity, food allergy, genetically modified soybean, mouse model, oral sensitization, Th2 response

Submitted 22 March 2005; revised 8 August 2005; accepted 27 November 2005

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Introduction

Food allergies are adverse reactions to an otherwise harmless food or food component that involves an abnormal response of the body's immune system to specific protein(s) in foods. Food allergies are caused by a wide

variety of foods. Theoretically, any food that contains protein would be capable of eliciting an allergic reaction, although foods vary widely in their likelihood of provoking allergic sensitization. Approximately 2–5% of the adult population is affected, with the prevalence among children being even greater [1]. The incidence of food

allergy around the world is increasing in line with other forms of allergic disease [2, 3]. Under healthy conditions, the immune system reacts towards innocuous dietary antigens by inducing antigen-specific systemic non-responsiveness, termed oral tolerance, whereas under pathological conditions, such as food allergy, it is believed that a dysregulation in the mechanisms of oral tolerance induction prevails [4, 5]. Most confirmed food allergies are associated with a relatively limited range of products (peanuts, crustaceans, fish, milk, eggs, tree nuts, wheat and soybeans) [6], although there are many other foods and food products that have also been implicated [7, 8]. Soybean is one of the major sources of protein in human and animal nutrition [9–11], and has also been well characterized as an important allergenic source [12]. At least 16 IgE-binding soy proteins with molecular masses from 7.5 to 97 kDa may be involved in clinical allergy [12]. The following proteins have been identified as major allergens: Gly m Bd 30K (thiol-protease P34), the storage proteins glycinin and β -conglycinin, profilin (Gly m 3), Gly m 2 and the Kunitz-trypsin inhibitor [13]. Moreover, recent data suggest an association between birch pollen and soy food allergy, because of Gly m 4, which has structural homology to Bet v 1 [14]. Therefore, patients with birch pollen allergy are at risk of experiencing allergic reaction to soy if they consume dietary products containing soy proteins. This is particularly important in some countries, like Central Europe, where birch is the major allergenic source but soy foods are not a common dietary component [15]. However, the relevance of soy as an allergen is growing in these countries because of a rising trend towards Asia foods, and to an increasing use of soy proteins in a number of foods and food products [9].

Advances in biotechnology have resulted in an increasing number of genetically engineered foods, and among these soybean is one of the most widespread. All genetically modified soybean grown in the world is represented by the Roundup Ready soybean that is resistant to glyphosate, the active ingredient in Roundup agricultural herbicides. This resistance was obtained by introduction of the glyphosate-tolerant *cp4 epsps* coding sequence, derived from the common soil bacterium *Agrobacterium* sp. strain CP4, into the soybean genome using particle-acceleration transformation [16, 17]. Globally, genetically modified soybean made up 61% of all transgenic crops grown in 2003 [18]. Foods produced through agricultural biotechnology should be assessed for their potential allergenicity. Although strategies exist for such assessments, improvements should be considered, especially in cases in which the gene of interest is derived from a source with no history of allergenicity. The FAO/WHO 2001 decision tree [19] proposes that informative data can be generated using animal models.

The purpose of this study was to develop and characterize the immune response in a murine model of soybean-

specific IgE sensitization induced by intragastrical immunization, in the presence of CT, with wild-type soybean extract (wt-SE), or with a genetically modified soybean extract (gm-SE). As the usual mouse diet includes soybean proteins, for our experiments we used Balb/c mice fed on soy-free feed, and born in our animal facilities from females fed on soy-free feed. Then, we used this model to compare the immunological response obtained both with wt-SE and with gm-SE, to assess the potential allergenicity of gm-SE with respect to its natural counterpart.

Material and methods

Soybean seeds

Soybean seeds were kindly provided by Dr Miraglia, Istituto Superiore di Sanità, Italy: 0% Roundup Ready Soybean (kindly supplied by an Italian importer) and 100% Roundup Ready Soybean (kindly provided by Monsanto) soybean lots. In order to verify the exact level of genetically modified soybeans in the provided materials, the two lots were analysed for their Roundup Ready Soybean content by PCR techniques in five test portions (100 mg each) by triplicate analyses. The genetically modified soybean content was less than 0.01% (limit of detection of the method) [20] in the first material, while for the 100% Roundup Ready Soybean, tested with 'real-time' PCR [21], the results were in the range of 95–102%.

Human sera

Human sera from 10 soybean-allergic patients (identified from A to L in Table 1) and from 5 non-atopic subjects (M–Q) were purchased from PlasmaLab International (Everett, WA, USA). All samples were collected from individuals living in the Everett area (WA, NW- United States). Each allergic donor was selected on the basis of clinical allergy to soybean, and was tested by Plasmalab with ImmunoCAP (Pharmacia, Uppsala, Sweden) for the presence of specific IgE to a wide panel of pollens, mites, foods, animal danders, insects and moulds (Table 1). All the patients were highly polysensitized, showing a number of IgE positivities against pollen, trees, foods, etc., the highest of which are reported in Table 1. Moreover, the presence of soybean-specific IgE was confirmed by in-house-performed ImmunoCAP analysis (last column in Table 1).

Preparation and characterization of soybean extracts

Soybean seeds were ground to a fine powder and extracted in PBS, pH 7.2, overnight (ON) at 4 °C, according to Eigenmann et al. [22], but excluding the final step of lyophilization. The extract was stored in aliquots at –80 °C. Soybean extracts (SEs) obtained either from

Table 1. Characteristic of human sera from soybean-allergic patients and healthy controls

Serum*	Age	Gender	Major allergen positivity reported by Plasmalab ^{†‡}	Soybean-specific IgE by in-house ELISA [§] (OD)	Soybean-specific IgE by in-house CAP (kUA/l)
A	44	m	D1, D3, E1, E2, F13, F14, G5, G6, H1, I6, K82, T3, W21	0.350	3.70
B	35	f	D1, D2, E2, E3, E5, F13, F14, G6, H1, I1, I3, K82, M1, M2, T3	0.311	3.60
C	40	f	D1, D2, E3, E5, F13, F14, G6, G8, H1, I1, I3, K82, M1, T2, T3, T4, w6, w9	0.728	8.23
D	29	f	D1, D2, D3, F13, F14, G5, G6, G8, H1, I1, I3, I6, M1, T3, w21	0.950	17.60
E	33	m	E2, E3, E5, F13, F14, G6, G8, H1, I1, I3, K82, M2, T3, W6, W9	0.178	2.25
F	43	m	D1, D2, D3, E1, E5, F13, F14, G5, G6, H1, I1, I3, K82, M1, M2, M10, T3, W6, W9, W21	0.884	17.90
G	47	f	D1, D2, E1, E2, E3, E5, F13, F14, G6, G8, H1, I1, I3, M11, T3, W6, W9	0.747	14.30
H	35	m	D1, D2, D3, E1, E2, F4, F5, F14, G5, G6, H1, I6, T3, W21	1.909	> 100
I	41	f	F1, F2, F9, F13, F14, M3	1.669	46.1
L	30	m	D1, D2, E3, E5, F13, F14, G6, G8, H1, I1, I3, K82, M4, M6, T3, W6, W9	0.729	8.52
M	35	m	–	0.046	–
N	46	m	–	0.036	–
O	29	f	–	0.057	–
P	30	f	–	0.081	–
Q	41	m	–	0.057	–

*Subjects were selected on the basis of clinical allergy to soybean and *in vitro* IgE evaluation. A–L, patients reporting soybean allergy; M–G, non-atopic subject.

[†]CAP values ≥ 3.5 kUA/L.

[‡]D1, *Dermatophagoides pter*; D2, *Dermatophagoides farinae*; D3, *Dermatophagoides* mix; E1, cat dander; E2, dog epithelium; E3, horse dander; E5, dog dander; F1, egg with; F2, milk; F9, corn, F4, wheat, F5, rye, F13, peanut, F14, soybean; G5, perennial rye grass; G6, timothy grass, G8, Kentucky blue; H1, house dust; I1, honey bee venom; I3, yellow jacket venom; I6, cockroach; K82, latex; M1, *Penicillium notatum*; M2, *Cladosporium herbarum*; M3, *Aspergillus fumigatus*; M4, *Mucor racemosus*; M6, *Alternaria tenuis*; M10, *Stemphylium botryosum*; M11, *Rhizopus nigricans*; T2, alder; T3, birch; T4, hazelnut; W6, mugwort; W9, English plantain; W21, *Parietaria judaica*.

[§]Cut-off value for the in-house ELISA is 0.050 OD, calculated on the basis of the mean OD value obtained with the normal group plus 3 SDs of the mean.

genetically modified organism (GMO)-free seeds (wt-SE) or 100% GMO seeds (gm-SE) were tested for qualitative analysis using the Trait RUR Lateral Flow Test (Strategic Diagnostic Inc., Newark, DE, USA), designed to detect the CP4 EPSPS protein. Test sensitivity is at least one RUR bean in 1000 GMO-free beans. From a quantitative point of view, the presence of CP4 EPSPS protein in the two SEs was evaluated by the GMO Soya ELISA Test Kit (Strategic Diagnostic Inc., Newark, NJ, USA) according to the manufacturer's instructions (detection limit for full-fat flour: 0.14%).

The protein content of the extract was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). SEs were analysed by SDS-PAGE under reducing conditions [23] and Coomassie brilliant blue (Bio-Rad Laboratories) staining and stored at -80°C until use.

The IgE-specific binding capacity of SEs was characterized by IgE immunoblotting and ELISA analysis as described previously [24], using a serum pool prepared from the 10 allergic subjects. In the IgE ELISA experiments, plates were coated with 20 $\mu\text{g}/\text{mL}$ of SEs and then incubated for 3 h at room temperature (RT) with serum samples diluted 1 : 5 in dilution buffer. Bound IgE were detected by a polyclonal horseradish peroxidase-conjugated goat anti-human IgE antibody (KPL, Gaithersburg, MD, USA) diluted 1 : 5000 and incubated ON at RT. In IgE Immunoblotting,

10 μg of proteins of SEs were loaded on a 15% SDS-acrylamide mini-gel. Proteins were then electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) ON at 80 mA. Strips were blocked in PBS–3% (w/v) gelatine (Bio-Rad, Richmond, Calif., USA) for 1 h and then incubated with sera diluted 1 : 3 in PBS/Tween ON at RT. Finally, the reaction was developed by incubation with ^{125}I -labelled anti-human IgE (Bio-Allergy, Rome, Italy), followed by autoradiography.

Animal and experimental protocol

The second-generation (F₂) female Balb/c mice born in our animal facilities, from females fed on soy-free feed (Mucedola s.r.l., Milan, Italy), were fed on the same soy-free feed and used in all the experiments. Mice used as controls were female Balb/c fed a commercial diet containing soybean meal. Animals were housed and treated according to the local guidelines for animal care (D.L. 116/92, which has implemented in Italy the requirements of the European Directive 86/609/EEC on laboratory animal welfare).

Mice were sensitized by intragastric (i.g.) administration of 1 mg of SE protein/mouse together with 10 μg /mouse, of cholera toxin (CT) (Inalco, Milan, Italy) as an

adjuvant in a total of 500 µL of an isotonic bicarbonate solution (B-saline), eight parts HBSS and two parts 7.5% sodium bicarbonate, to neutralize stomach acidity [25], on days 0, 7 and 21. Control animals received an equal volume of B-saline alone, or an equal quantity of SE without CT, or CT alone. Mice were bled from the retro-orbital plexus on days 0, 7, 14, 21, 28 and 35. All samples are stored at -20°C until analysis.

ELISA, ELISA inhibition

Microtitre plates (Greiner Bio-One, Frickenhausen, Germany) were coated with the SEs or with the irrelevant pollen extracts from *Parietaria judaica* (PjE) and *Olea europaea* (OeE) used as controls (20 µg/mL) in carbonate/bicarbonate buffer, pH 9.6, for 3 h at 37°C and ON at 4°C , respectively. Unless otherwise indicated, serum samples were diluted in phosphate-buffered saline (PBS), 0.5% w/v BSA, 0.05% v/v Tween-20 and plates were washed between each incubation step with PBS, 0.05% v/v Tween-20. Non-specific-binding sites were blocked with PBS, 1% w/v BSA for 2 h at RT. Serum samples were added in duplicate (diluted 1:20 for IgE measurement, 1:40 for IgG, IgG1 or IgG2a) and incubated ON at 4°C in the IgE ELISA or 2 h at RT in the IgG ELISA. Pre-immune sera from each individual mouse were used in parallel as controls. After washing, bound antibodies were detected by adding a peroxidase-labelled rat monoclonal anti-mouse IgE (BD Biosciences Pharmingen, San Diego, CA, USA) for 5 h at RT, or peroxidase-labelled rat monoclonal anti-mouse IgG, IgG1, and IgG2a for 1 h at RT. All the secondary antibodies were diluted 1:500. The peroxidase substrate (ortho-phenylenediamine chloride, OPD; Sigma-Aldrich, Milan, Italy) was then added, and absorbance was determined with an ELISA reader (Bio-Rad Laboratories) at 490 nm. In the IgE ELISA inhibition experiments, plates were coated with wt-SE or with gm-SE as described previously, and incubated with a pool of sera, obtained either from mice sensitized with wt-SE+CT or from mice sensitized with gm-SE+CT, which was pre-incubated for 3 h at RT with decreasing concentrations of the SEs (or the irrelevant pollen extracts PjE and OeE) used as inhibitors (from 100 to 0.01 µg/mL) in dilution buffer. Binding of IgE was then evaluated as described above.

Spleen cell proliferation

Two weeks after the last immunization, mice were killed and individual spleens were aseptically removed and minced and cell suspensions were prepared. After erythrocyte lysis, spleen cells were resuspended in complete medium (RPMI 1640 containing 10% fetal bovine serum, 2 mM L-glutamine, 25 mM Hepes, 100 IU/mL penicillin, 100 mg/mL streptomycin) and counted. Proliferation assays were performed by plating cell suspensions at a

concentration of 2×10^5 /well in 96-well round-bottomed plates (Costar, Corning Life Sciences, Acton, MA, USA) in triplicate, and stimulating the cells with 50 µg/mL of wt-SE, gm-SE, PjE, OeE or medium alone as controls. Tritiated thymidine (1 mCi/well) (ICN Pharmaceuticals, Biomedical Division, Milan, Italy) was added on day 6, and cells were harvested 18 h later on Microbeta filter mats using a 96-well harvester. The stimulation index (SI) was calculated with the following formula: mean c.p.m. in allergen-stimulated cultures/mean c.p.m. in controls.

Quantification of cytokines

Individual spleens were removed 2 weeks after the last i.g. administration of the antigen. Cells were isolated and suspended in complete medium as described above. Cell suspensions were cultured in 24-well plates 2×10^6 /well/mL in the presence or absence of wt-SE or gm-SE (50 µg/mL). The supernatants were collected after 7 days of culture. Levels of cytokines IFN- γ , IL-4 and IL-5 were determined by ELISA, according to the manufacturer's instructions (BD Biosciences Pharmingen).

Statistical analysis

Differences between values were analysed by the Mann-Whitney rank sum test and the Wilcoxon signed-ranks test. *P*-values < 0.05 were considered statistically significant.

Results

Immunochemical characterization of SEs

The results obtained by the qualitative and quantitative evaluation of the content of CP4 EPSPS in the two SEs (Trait RUR Lateral Flow Test and GMO Soya ELISA Test Kit) confirmed that wt-SE was GMO free, whereas gm-SE was GMO positive. In the quantitative assay, the gm-SE at the highest dilution suggested by the manufacturer's instructions (1:300) was found to contain CP4 EPSPS in a proportion of $> 2.5\%$ of the total protein content.

SDS-PAGE patterns of wt-SE and gm-SE are shown in Fig. 1 (panel A). Multiple bands ranging from 14 to 98 kDa can be identified by Coomassie Blue staining, and give comparable patterns for both extracts.

The presence of allergenic components recognized by human IgE in the SEs was studied by using 10 sera from soybean-allergic patients. All the 10 sera from soybean-allergic patients displayed OD₄₉₀ values significantly higher than the values shown by the normal group in an ELISA test (cut-off 0.050 OD) (Table 1).

Furthermore, immunoblotting analysis, performed with a pool of the same 10 allergic sera, showed that IgE-specific binding proteins are present in both the SEs

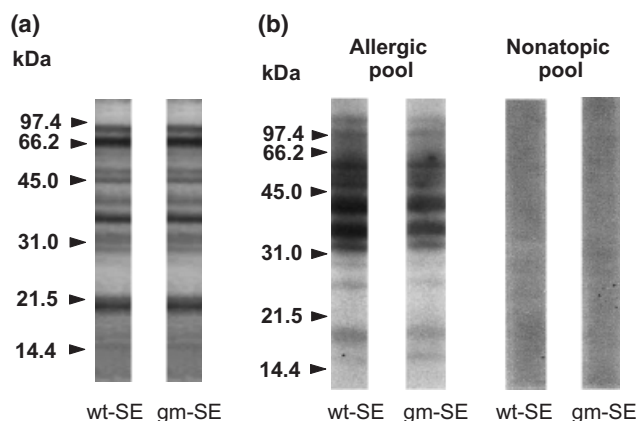


Fig. 1. Characterization of wild-type soybean extract (wt-SE) and genetically modified soybean extract (gm-SE). (a) SDS-PAGE pattern of wt-SE and gm-SE, stained with Coomassie brilliant blue. (b) Human IgE reactivity profiles to nitrocellulose-blotted wt-SE and gm-SE. Nitrocellulose strips were incubated with a pool of 10 allergic sera or with a pool of five non-atopic subjects.

(Fig. 1b). Prominent IgE-binding bands could be observed at 16–21, 25, 30, 35–40, 50, 60 and > 95 kDa. No IgE binding could be detected with the control sera.

Mice sensitized both with wt-SE and gm-SE produce high levels of specific IgE and IgG1 antibodies

To set-up the experimental protocol, we preliminary tested pooled serum samples from F_2 progeny, i.g. sensitized with wt-SE with or without CT, for the presence of wt-SE-specific IgE antibodies. As shown in Fig. 2a, only the administration of wt-SE in association with CT was able to induce wt-SE-specific IgE antibody, reaching a maximum level on day 28. Control mice, immunized with CT alone or with B-saline, did not show any wt-SE-specific response (Fig. 2a). Irrelevant proteins (e.g., PjE and OeE allergens from pollen extracts) were used as antigen in a direct IgE ELISA to test specificity, and were not recognized by immune sera (data not shown). Balb/c mice bred on a commercial diet and sensitized by the same protocol did not produce a specific IgE response (Fig. 2b).

Having established the sensitization protocol, we measured allergen-specific IgE and IgG responses in the mouse sera obtained from animals treated with three i.g. sensitization either with wt-SE or with gm-SE on days 0, 7 and 21. We found high levels of specific IgE, IgG and IgG1 antibodies when wt-SE was used as antigen, and the sera were from mice sensitized by homologous allergen (Fig. 3, panel A, B, C, respectively), whereas a low production of soybean-specific IgG2a was observed (Fig. 3d). The same result was obtained when gm-SE was coupled on the plate and sera were obtained from mice sensitized by the same allergen (Fig. 3). These findings are indicative of a Th2-type response induced by this regimen of sensitization. No

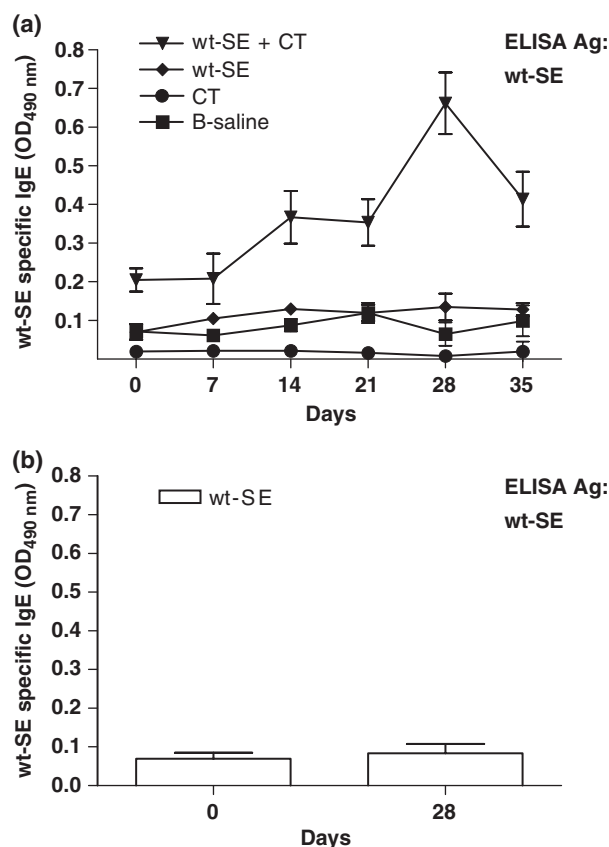


Fig. 2. IgE response induced in F_2 mice after immunization with soy allergens. (a) Time-response analysis of specific IgE response to wild-type soybean extract (wt-SE) in pooled sera from mice sensitized with wt-SE + cholera toxin (CT) (▼), wt-SE (◆), B-saline (■) or CT alone (●). (b) Specific IgE response to wt-SE in Balb/c mice bred on a commercial diet (with soy) and sensitized with wt-SE+CT, measured on days 0 and 28. Results are expressed as the mean of three experiments $\pm$ SEM.

significant differences were found when we compared the specific IgE or IgG antibody responses, induced in mice after i.g. administration of wt-SE proteins, with that obtained with gm-SE proteins by the same route.

wt-SE and gm-SE share the same allergenic structures

On the basis of this observation, we then analysed by IgE ELISA inhibition assays whether wt-SE and gm-SE shared the same allergenic structures. Results obtained with pooled sera from mice sensitized either with wt-SE or with gm-SE are shown in Fig. 4. gm-SE was able to inhibit the binding of wt-SE-specific IgE when tested against wt-SE (Fig. 4a), and the amount of gm-SE required to produce 50% inhibition was the same as the amount of the homologous (wt-SE) inhibitor (6.6 vs. 7.2 μ g/mL). A similar result was obtained when gm-SE was used as an antigen on the plate and the pooled sera were obtained from mice treated with gm-SE, (Fig. 4b). In both cases, it was possible to achieve 100% inhibition. Irrelevant

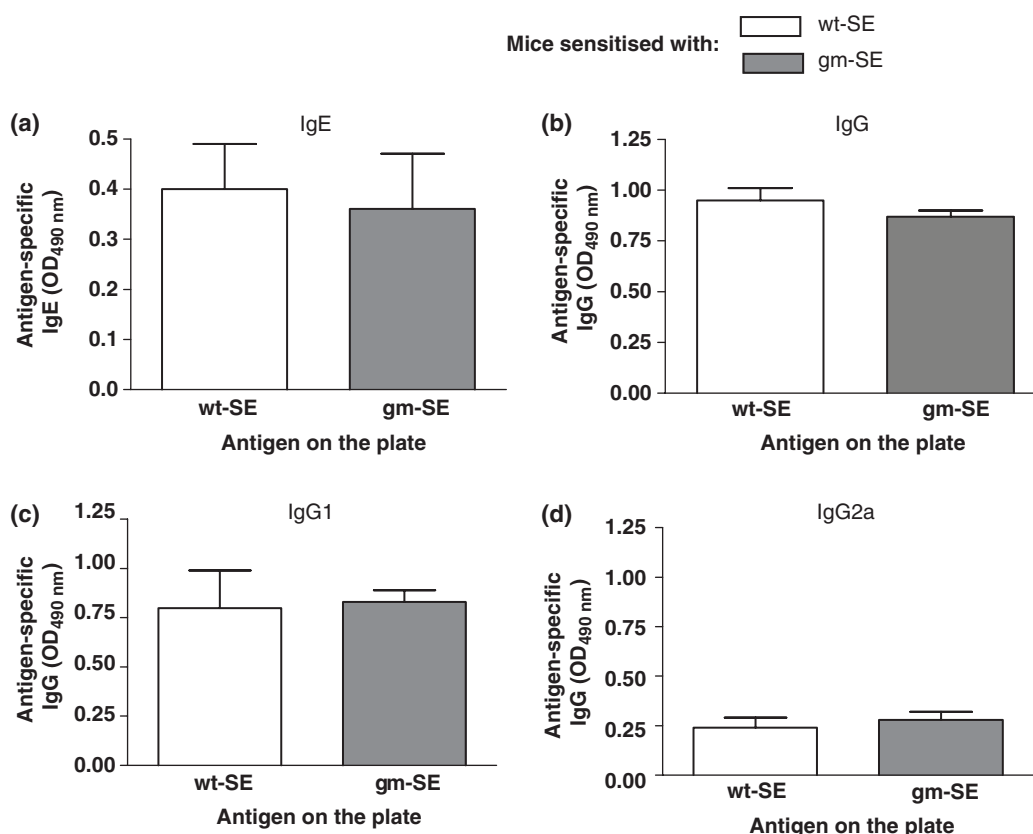


Fig. 3. Allergen-specific IgE and IgG response induced in F_2 mice sensitized with soybean extracts (SEs). Specific IgE (a), IgG (b), IgG1 (c) and IgG2a (d) responses to wild-type soybean extract (wt-SE) or to genetically modified soybean extract (gm-SE) in mice sensitized with wt-SE (white histograms) or with gm-SE (black histograms). A representative experiment out of six is shown. Results are expressed as mean of individual sera from 10 mice/group $\pm$ SEM.

proteins (e.g., PjE and OeE) were used as control inhibitors and were not able to inhibit the soybean-specific IgE response. Altogether, these results show that wt-SE and gm-SE induce a specific IgE response and share the same allergenic structures. Therefore, in this animal model, sensitization with wt-SE or with gm-SE was able to induce an equivalent allergen-specific IgE response.

*Sensitization with wt-SE or with gm-SE induces similar *in vitro* proliferative responses to either wt-SE or gm-SE*

Finally, we characterized the proliferative response both in mice sensitized with wt-SE and in mice sensitized with the gm-SE. Antigen-specific proliferation was detectable only in mice sensitized with the antigen + CT. We evaluated antigen-specific proliferation of spleen cells isolated from mice immunized with wt-SE or from mice immunized with gm-SE (Fig. 5a). Spleen cells were stimulated *in vitro* with wt-SE or with gm-SE, and with PjE and OeE as negative controls (data not shown).

The proliferative response induced *in vitro* by gm-SE on gm-SE primed spleen cells was comparable with that stimulated by wt-SE on spleen cells from mice immunized

with wt-SE (stimulation indices ranging from 32.0 to 63.0 with gm-SE ($P=0.683$), and from 23.0 to 68.6 for wt-SE ($P=0.537$)).

Moreover, a comparable proliferative response was obtained after spleen cell stimulation with the homologous as well as with the heterologous extract used for mice immunization.

Taken together, these results suggest that gm-SE share the relevant T cell epitopes of wt-SE.

T cell cytokine profile induced by wt-SE- or gm-SE-specific stimulation

To determine the polarization pattern of the T cell response, the cytokines produced by spleen cells were measured in culture supernatants. As shown in Fig. 5b, *in vitro* stimulation with the homologous antigen used for mice sensitization induced high levels of IL-4, IL-5 and IFN- γ . Undetectable levels of cytokines were found in culture supernatants of unstimulated cells.

The production of high levels of IL-4 and IL-5 by spleen cells from sensitized mice after *in vitro* stimulation with both allergenic extracts, together with the predominant

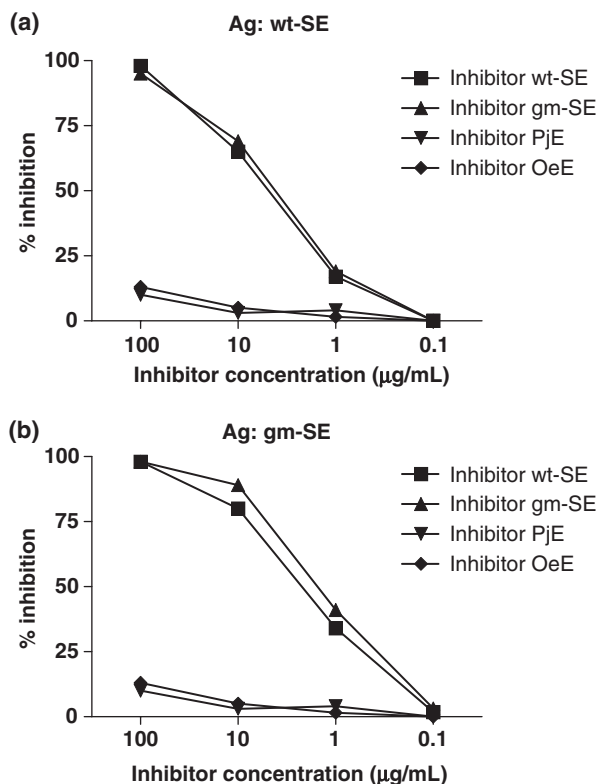


Fig. 4. Specific IgE ELISA inhibition to wild-type soybean extract (wt-SE) (a) and genetically modified soybean extract (gm-SE) (b) coated to the plates, by wt-SE (■), gm-SE (▲), PjE (▼) and OeE (◆) used as inhibitors. Pooled sera were obtained from mice sensitized with wt-SE (a) or with gm-SE (b). A representative experiment out of three is shown.

production of IgE and IgG1 in their serum, is compatible with the idea that intragastric immunization with SEs in the presence of CT prevalently causes a Th2-oriented response to these allergens. In addition, the Th1 cytokine IFN- γ was induced by sensitization, but never in the absence of Th2 cytokines (i.e., IL-4 or IL-5).

Discussion

With the development of genetically modified crop plants there has been a growing interest in the approaches available to assess the potential allergenicity of novel gene products [26–28]. This allergenicity might derive from changes in endogenous protein levels, expression of known allergens in different foods and the expression of novel proteins that may be allergenic. A report in 2001 of a joint FAO/WHO expert consultation on allergenicity of foods derived from biotechnology suggests an integrated and stepwise, case-by-case approach, which incorporates consideration of the serological identity of the protein of interest with known human allergens, examination of the amino acid homology with, and/or structural similarity to, allergenic proteins and measurement of the stability of the protein in a simulated gastric fluid [19]. The same document

gave an indication that, for additional assessment of the potential allergenicity of expressed proteins, informative data can be generated using animal models. These data could be used in concert with the approaches summarized above. Such models would be of considerable benefit if they facilitated a more direct evaluation of the ability of proteins to *in vivo* induce allergic responses. Methods have been described based upon measurement of immune responses induced in rats and in mice, either in the presence or in the absence of an adjuvant, following oral or intraperitoneal administration of protein [29–41].

Soybean protein is used in a number of food products but is also a common cause of food allergy. Several soybean allergens have been isolated from the protein fraction of seeds and from lecithins [22, 42–45]. However, to our knowledge, there are no reports on the development of animal models of soybean food allergy. A recent study from Christensen et al. [46] has shown that Balb/c mice fed a soy-containing diet generate a non-allergic antibody response towards the same soy proteins known to be allergenic in humans. The Balb/c mouse has been widely utilized to evaluate the sensitizing potential of novel proteins. An advantage of this model is that the Balb/c mouse strain is known to favour the development of Th2-type immune responses and the production of IgE antibody. Although it has been argued that, in the context of assessing the safety of dietary proteins, oral administration is the preferred route of exposure, it has been demonstrated that such a regimen may lack the sensitivity required for effective identification of the inherent sensitizing potential. This is probably attributable, at least in part, to the fact that oral exposure to the antigen generally results in the development of tolerance [4]. Therefore, the major general obstacle towards establishing food allergy models is the strong innate tendency of animals to develop immunological tolerance to ingested antigens. This is particularly relevant in the case of soybean allergen, as the usual mouse diet includes soybean proteins.

Recent studies have developed murine models of IgE-mediated food allergy based on oral coadministration of antigen with an optimal dose of CT, to generate a robust allergen-specific antibody response [34–36, 47–49]. CT, a potent mucosal adjuvant, has been shown to activate Th2 cells and promote the production of IL-4 and IgE antibodies [25, 50].

In the present study, we used a similar approach to develop our murine model, combining it with the use of Balb/c mice of the second (F2) offspring generation bred on a soy protein-free diet, and born in our animal facilities from mothers fed on a soy-free feed. We generated the first murine model of IgE-mediated soybean sensitization, in which a soybean-specific IgE response was induced by oral immunization, and reported the characterization of the antigen-specific cellular and humoral responses. Then, to assess the potential allergenicity of gm-SE with respect

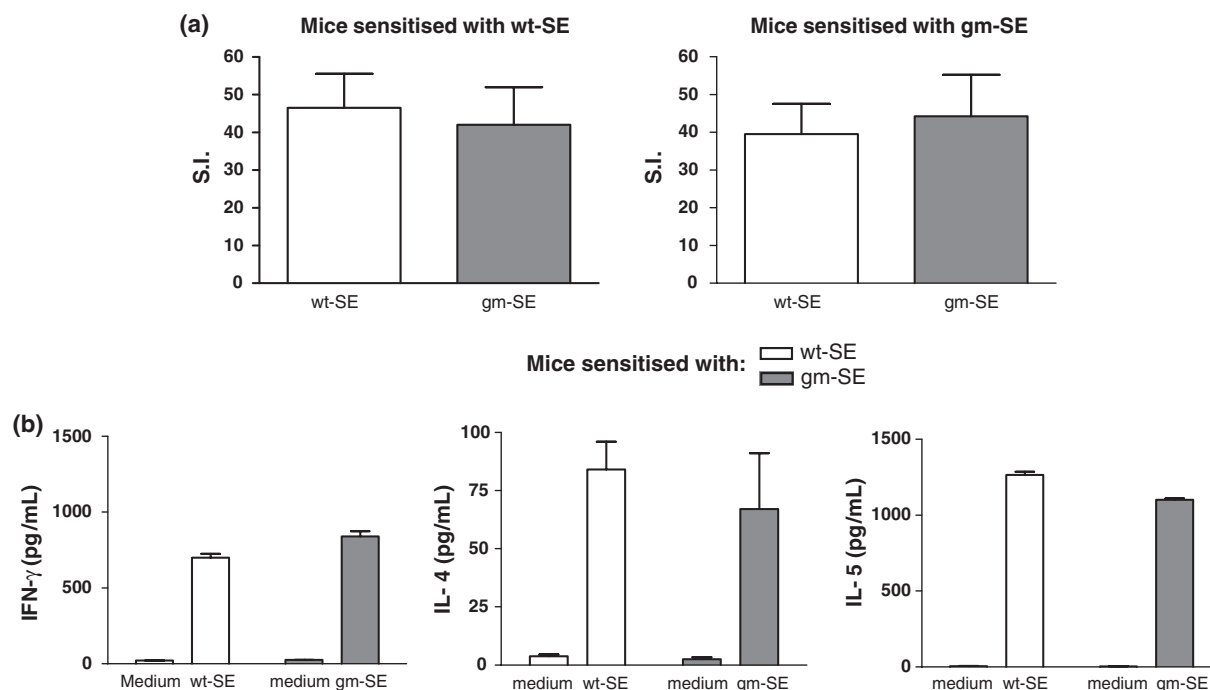


Fig. 5. Proliferation of spleen cells from mice sensitized with wild-type soybean extract (wt-SE) or genetically modified soybean extract (gm-SE) (a) and stimulated *in vitro* with wt-SE (white histograms) or gm-SE (black histograms). Results are expressed as mean stimulation index (SI) $\pm$ SEM obtained with 10 individual mice/group. A representative experiment out of six is shown. (b) IL-4, IL-5 and IFN- γ production of spleen cells from mice sensitized with wt-SE (white histograms) or gm-SE (black histograms), after *in vitro* stimulation with the homologous antigen. Results are expressed as mean of values $\pm$ SEM obtained with 10 individual mice/group. A representative experiment out of six is shown. Quantitation limits for the ELISA assays were: < 15 pg/mL for IFN- γ and < 5 pg/mL for IL-4 and IL-5.

to its natural counterpart, we used this model to compare the immunological response obtained with wt-SE or with gm-SE.

To this aim, two allergenic extracts from wt-soybean and from gm-soybean seeds were prepared and also characterized by means of SDS-PAGE, followed by IgE ELISA and immunoblotting. In preliminary experiments, the optimal extraction conditions of wt-soybean and of gm-soybean seeds were established. The final protocol was chosen on the basis of two prevalent criteria: (1) to optimize extraction of CP4 EPSPS protein from gm-soybean seeds; and (2) to obtain a material representative of all the components present in the primary allergenic source (e.g., soybean seed).

SDS-PAGE analysis of the SEs demonstrated many different protein components but the protein pattern did not show differences between wt-SE and gm-SE. It was not possible to identify in the gm-SE electrophoretic pattern a particular band in the molecular weight region corresponding to the CP4 EPSPS protein (46 kDa). This finding can be explained assuming that the CP4 EPSPS protein migrates in a region in which several components with similar molecular weight comigrate. However, the presence of CP4 EPSPS protein in the gm-SE was confirmed by a qualitative and quantitative analysis using a Trait RUR Lateral Flow Test and a GMO Soya ELISA Test

Kit, respectively. The presence of the *cp4-epsps* gene in gm-soybean seeds was assessed by 'real-time' PCR.

gm-SE and wt-SE were characterized by ELISA using serum samples from 10 subjects having soybean-specific IgE antibody, as confirmed by ImmunoCAP. Immunoblotting analysis with the pool of 10 sera showed the presence of a number of distinct IgE-binding proteins ranging from 14 to > 95 kDa in both extracts, which were comparable with the IgE recognition pattern described previously in the literature for SE [12]. The immunoblotting reactivity of individual sera showed different frequency of IgE binding to soybean proteins migrating in the following MW areas: < 14 (four out of 10 sera), 16–18 (4/10), 20–25 (3/10), 30 (10/10), 35 (5/10), 50–70 (9/10) and > 95 (8/10) kDa (data not shown). No discernible differences were found in the recognition of the gm-SE with respect to wt-SE by human IgE from allergic patients, as already demonstrated by Burks and Fuchs [51] in their study on the same gm-soybean variety. Nevertheless, a commentary to the same study [52] underlined the importance of evaluating the allergenic potential of newly introduced proteins in animal models.

The SEs, characterized by the presence of CP4 EPSPS and of the relevant IgE-binding proteins, were then used to establish a murine model of IgE sensitization to soybean. We immunized Balb/c mice with both wt-SE

and gm-SE and obtained high levels of specific IgE and IgG1 vs. low levels of IgG2a. This pattern is indicative of a Th2-type response induced by the oral immunization with CT as an adjuvant. The levels of IgE and IgG1 antibodies produced by gm-SE-sensitized mice were comparable to those obtained from wt-SE-sensitized mice. Moreover, IgE antibodies produced after immunization with wt-SE were able to inhibit completely the binding of IgE obtained by oral administration with gm-SE. Similar results were obtained in the opposite case. These data strongly suggest that gm-SE shared the same allergenic components with wt-SE. In particular, the complete IgE inhibition (up to 100%) obtained in the assay performed on gm-SE as an antigen, wt-SE as an inhibitor and sera from gm-SE-sensitized mice sustains the hypothesis that the exogenous CP4-EPSPS protein is not able to induce an IgE response under our conditions.

T lymphocytes and cytokines have been demonstrated to play a pivotal role in the induction of IgE response. Therefore, to further assess the suitability of our animal model for testing the allergenicity of genetically modified foods, we evaluated the T cell responses to the SEs. We found that spleen cells from mice treated with gm-SE exhibited the same proliferative responses to wt-SE *in vitro* stimulation compared with homologous antigen stimulation and *vice versa*. Antigen stimulation of spleen cells from mice sensitized either with wt-SE or with gm-SE induced significantly increased and comparable production of IL-4, IL-5 and IFN- γ .

Taken together, our data on the humoral and cell response demonstrate that in sensitized mice, we observed a predominantly Th2-type immune response, with increased food-specific IgE and IgG1 antibodies and concomitant production of IL-4 and IL-5. Similar antibodies and cytokine profile have been found in human beings with food allergy. It has been well documented that Th1/Th2 cytokines are responsible for the production of different antibody isotypes in mice, e.g., IL-4 drives IgE and IgG1, and IFN- γ favours IgG2a [53, 54]. The Th1 cytokine IFN- γ was induced by sensitization, as observed in other murine models [55, 56], but did not result in IgG2a production. Its presence is consistent with the findings of Hofman [57], who reported that serum IL-4 levels were twice as high, and IFN- γ levels four times as high in children with food allergy than in control subjects. However, our results, like those found by other authors [55, 56], cannot be fully explained by the Th1/Th2 paradigm and rather suggest that other non-Th1/Th2 pathways may also be important.

We could also show that gm-SE induced an immunological response comparable with that induced by wt-SE. Moreover, results obtained in our model by specific IgE ELISA inhibition and by antigen-specific T cell proliferation demonstrated that wt-SE and gm-SE shared B and T epitopes. Finally, the absence of humoral and cell

response against control proteins in the same assays confirms that our model is specific for the components of SE.

In conclusion, considering that there is no single predictive bioassay available to assess the allergenic potential of proteins in humans [19], the murine IgE sensitization model described here provide valuable information regarding the allergenicity of modified soybean derived from biotechnology, and it could be a suitable system for the *in vivo* testing of genetically modified foods.

Acknowledgements

We thank Dr. ssa Monica Boirivant, Dr. ssa Mariarosaria Marinaro and Dr. Carlo Pini for their valuable contributions. We also thank Dr. Rodolfo Lorenzini for his helpful advice. The skilful technical assistance in animal management by Mr Antonio Di Virgilio and Mr Agostino Eusepi is greatly acknowledged. This work was supported in part by grant n. OAC/F8 from the Italian Ministry of Health.

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