

Soy immunotherapy for peanut-allergic mice: Modulation of the peanut-allergic response

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Background: Allergen-specific immunotherapy (IT) is an effective therapeutic modality to prevent further anaphylactic episodes in patients with insect sting hypersensitivity and is being investigated for peanut allergy. So far, peanut-specific IT has been unsuccessful because of the side effects of therapy. Soybean seed storage proteins share significant homology with the respective peanut allergens.

Objective: This study was undertaken in mice to investigate whether specific doses of soybean would desensitize peanut-allergic mice.

Methods: C3H/HeJ mice were sensitized to peanut with 3 intraperitoneal (IP) injections of crude peanut extract. The mice were desensitized by IP injections with either crude peanut or soybean extract for 4 weeks, 3 times a week. Controls included placebo desensitization with PBS and naive mice.

After 2 weeks of rest, mice were challenged IP with crude peanut extract. Thirty minutes later, symptom scores and body temperatures were recorded. Serum immunoglobulins, peanut-induced splenocyte proliferation, and secreted cytokines were measured before and after desensitization.

Results: The clinical symptoms in the soybean- and peanut-desensitized animals were markedly reduced compared with the placebo-treated mice. Specific IgG1 levels to crude peanut were significantly lower in the soy IT group than in the peanut IT group. The cellular response to crude peanut was also downregulated in the soy IT group, as shown by decreased peanut-specific stimulation indices and a cytokine profile skewed toward a T_H1 response.

Conclusions: Soy IT can be used to desensitize/downregulate peanut-specific response in peanut-allergic mice and could provide a new therapeutic intervention for peanut allergy. (*J Allergy Clin Immunol* 2004;114:915-21.)

Key words: Food allergy, peanut hypersensitivity, immunotherapy, cross-reactivity, soybean, legumes, animal model

Food-allergic reactions have generated increasing concern in the United States, with approximately one fourth of

Abbreviations used

CPE: Crude peanut extract
CSE: Crude soybean extract
IP: Intraperitoneal
IT: Immunotherapy
PN: Peanut

American households altering their dietary habits because a member of the family is perceived to have food allergies.¹ It is interesting to note that prospective studies have indicated that only 6% to 8% of children younger than 4 years of age experience IgE-mediated food-allergic reactions, with approximately 1.5% of young children reacting to cow's milk, approximately 1.3% to hen's egg, and 0.5% to peanut.^{2,3} The prevalence of food hypersensitivity in adults is reportedly less common, but a recent survey in the United States found that 1.3% of adults are allergic to peanuts or tree nuts.⁴ Given the estimated frequency of allergy to fish, shellfish, and other sensitivities, it is likely that approximately 2% of the adult population, or approximately 5.5 million Americans, are affected by food allergies.⁵

Despite increased recognition and understanding of food allergies, food-induced anaphylaxis is the single most common cause of anaphylaxis, accounting for approximately one third of anaphylaxis cases seen in hospital emergency departments.⁶ It is estimated that approximately 30,000 food-induced anaphylactic events are seen in US emergency departments each year and that approximately 200 fatal cases occur in the United States each year. Peanuts and tree nuts account for more than 80% of these reactions.⁶

Peanut allergy is one of the most serious of the immediate hypersensitivity reactions to foods in terms of the persistence and severity of the reaction, and it seems to be a growing problem.^{4,6} Because of the persistence of this reaction and the lack of effective treatment, allergen-specific immunotherapy (IT) is currently being examined as a treatment option. An understanding of the molecular mechanisms of allergen-specific IT is vital to ensure the eventual successful treatment of patients allergic to peanuts. Previous attempts to use peanut-specific IT have been unsuccessful because of the side effects of therapy.⁷

Because the seed storage proteins in soybeans share considerable amino acid homology with their respective peanut allergens, we used soybean IT in our peanut-allergic mouse model to downregulate the peanut-allergic

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responses. Our hypothesis was that soybean IT would desensitize an ongoing allergy to peanuts in the mouse model, which could be extrapolated to patients with peanut-allergic reactions. The soybean IT desensitization would benefit patients by decreasing the likelihood that they would have life-threatening allergic reactions to "hidden allergen" sources of peanuts in other foods or, possibly, by allowing other types of peanut IT to be used more safely. Our specific aim was to investigate the immunoglobulin levels and peanut-specific T-cell responses in peanut-allergic mice after desensitization with soybeans.

We conducted a preliminary study in an established peanut-allergic mouse model^{8,9} to determine the feasibility of using soybean IT to desensitize animals with peanut allergy. The soybean IT-treated animals had a significant reduction of clinical symptoms after peanut challenges compared with placebo-treated animals. If soybean IT can be used in patients allergic to peanuts and is effective, the treatment would provide an immediate therapeutic option for patients with peanut hypersensitivity.

METHODS

Mice and reagents

Five-week-old female C3H/HeJ mice purchased from The Jackson Laboratory (Bar Harbor, Me) were maintained on soy- and peanut-free chow (Harlan Teklad, Madison, Wis) under specific pathogen-free conditions. Standard guidelines for the care and use of animals were followed.¹⁰ Crude peanut extract (CPE) and crude soybean extract (CSE) were both prepared from defatted raw flours. Briefly, the flour (1:10, wt/vol) was extracted in 10× PBS overnight at 4°C. After centrifugation at 30,000g for 60 minutes, the supernatant was filter-sterilized, measured for protein concentration using the BCA method (Pierce, Rockford, Ill), and stored as aliquots at -20°C. By using this extraction procedure, the percentages of the major allergens Ara h 1 and Ara h 2 in the crude extract were 14% and 6%, respectively, as estimated by densitometric scanning of a Coomassie blue-stained polyacrylamide gel after denaturing electrophoresis. Aluminum hydroxide was used as adjuvant (Imject Alum, Pierce).

Intraperitoneal antigen sensitization, desensitization, and challenge

Peanut sensitization and challenge were adapted from a previously described protocol except that sensitization was performed by intraperitoneal (IP) administration of 0.5 mg of CPE in PBS together with 2.0 mg of Alum (0.5 mL injected) on days 1, 7, and 21.⁸ Desensitization was started 2 weeks after the last sensitization dose and was given IP (0.5 mL injected) 3 times per week for 4 weeks. The doses were 0.1, 0.25, 0.5, and 0.5 mg of CPE for the peanut-desensitized group and 0.25, 0.5, 1.0, and 1.0 mg of CSE for the soybean-desensitized group. For negative controls, sham-desensitized mice received PBS, and naive mice received only PBS. Challenges with CPE (1.0 mg in 0.5 mL of PBS) were conducted IP 2 weeks after the last desensitization dose. Sensitization to peanut was assessed 1 week after the third injection by challenging and killing a subgroup of mice.

Assessment of hypersensitivity reactions

Anaphylactic symptoms were evaluated 30 minutes after the challenge dose by using a scoring system from a previous report⁸: 0,

no symptoms; 1, scratching and rubbing around the nose and head; 2, puffiness around the eyes and mouth, diarrhea, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate; 3, wheezing, labored respiration, cyanosis around the mouth and the tail; 4, no activity after prodding, or tremor and convulsion; and 5, death. Scoring of symptoms was performed in a blinded manner by 3 independent investigators.

Measurement of core body temperatures

Thirty minutes after challenge and after scoring the symptoms, temperatures were measured with a rectally inserted thermal probe (Physitemp Instruments Inc, Clifton, NJ).

Measurement of serum peanut-specific IgG1 and IgG2a and total IgE levels

Tail vein blood was obtained during sensitization and treatments and after desensitization. Levels of peanut-specific IgG1 and IgG2a and total IgE were measured by ELISA as described previously⁹ with minor modifications. Plates were coated with CPE at 20 µg/mL, and sera were diluted at 1:8000. Detection was performed with horseradish peroxidase-conjugated goat antibodies anti-mouse IgG1 and IgG2a (SouthernBiotech, Birmingham, Ala) at the working dilutions of 1:40,000 and 1:20,000, respectively. The peroxidase activity was measured with 3,3',5,5'-tetra-methylbenzidine (Kirkegaard and Perry, Gaithersburg, Md) at 450 nm. For the total IgE assay, serum dilutions of 1:60 and color development with 3,3',5,5'-tetra-methylbenzidine were the only adjustments from the original protocol. For each mouse, the immunoglobulin concentrations were the mean of triplicates.

Proliferation assays and quantification of secreted cytokines

Splenocytes were isolated from pooled spleens removed from nonchallenged mice (n = 5) of each group (peanut-sensitized without desensitization group, peanut-desensitized group, soybean-desensitized group, sham-desensitized mice, and naive mice) and plated for the different assays as detailed in a previous report.⁹ Cells were stimulated with CPE (100 µg/mL) or PHA (20 µg/mL). Levels of IFN-γ and TNF-α in the supernatants of 24-hour cultures were determined by flow cytometry using the Cytometric Bead Array (BD Pharmingen, San Diego, Calif).

Statistics

Statistical analyses were performed by using SPSS 11.5 for Windows (SPSS Inc, Chicago, Ill). For continuous outcomes, pairwise comparisons between group means were made by using 2-sample *t* tests with unequal variance. Group differences in the discrete scale symptom scores were assessed with Mann-Whitney *U* tests. All tests were conducted at α = 0.05. Because of the preliminary nature of this research, no adjustment was made for multiple comparisons.

RESULTS

This study was undertaken in mice to investigate whether specific doses of soybean would desensitize peanut-allergic mice. Twice as much crude soybean (1.0 mg of CSE) as peanut (0.5 mg of CPE) was used for desensitization in this study, a dose based on previous experiments. The clinical effects of IT on anaphylactic reactions were measured after peanut challenge, and

peanut-specific antibody levels and cytokine profiles were determined.

It is noteworthy that injection of the third dose of peanut during the sensitization phase induced strong anaphylactic symptoms (scores from 3 to 4) in most animals. However, no symptoms were noticed after injection of either peanut or soybean during the desensitization period.

As can be seen in Fig 1, the symptom scores after the peanut challenge were very similar in the peanut IT-treated and soy IT-treated groups (symptom scores of approximately 1) but were significantly higher in the PBS IT-treated mice (symptom scores of approximately 4). In addition, the core body temperature (Fig 2) was not statistically different between the peanut and soy IT groups but was significantly lower in the PBS-treated mice (PN vs PBS, $P < .01$; soy vs PBS, $P < .01$).

In this study, the associated peanut-specific antibody levels and splenocyte cytokine levels were similar to those seen in previous studies with peanut IT. The antibody levels of peanut-specific IgG1 in the soy IT group were significantly less than those in the peanut IT group, and both had levels significantly lower than those of the sham-desensitized group (Fig 3). Peanut and soy IT efficiently interrupted the significant increase in specific IgG1 concentration that occurred between the end of the sensitization and desensitization phases (PN vs sham-desensitized mice, $P < .05$). Regarding peanut-specific IgG2a and total IgE levels, no significant differences were found between the 2 IT groups (data not shown).

In investigating the immune changes with peanut and soy IT, we examined peanut-specific cellular proliferation (stimulation index). In Fig 4, the stimulation indexes for the peanut and soy IT groups (cells pooled from 5 animals in each group) were not different from each other but were markedly lower than those of the PBS IT-treated animals. Representative of the cellular changes that occurred after IT, the levels of IFN- γ and TNF- α (Figs 5 and 6) in both the peanut IT-treated and soy IT-treated animals were notably higher than in the PBS IT-treated animals.

DISCUSSION

At the present time, strict avoidance of food allergens and ready access to self-injectable epinephrine is the standard of care for food allergy. Unfortunately, for a ubiquitous food such as peanut, the possibility of inadvertent ingestion is great. It is estimated that more than 50% of individuals who are allergic to peanuts will have an accidental reaction to peanuts over a 2-year period.⁴

Allergen IT is an effective therapeutic modality to prevent further anaphylactic episodes in patients with insect sting hypersensitivity when they have experienced significant systemic symptoms.¹¹ Because allergen IT can downregulate the specific IgE response and the cellular response to allergens, treatment with IT is now being studied as a possible option for food allergy.⁷ The goal of this type of peanut-specific IT is not for patients to eat

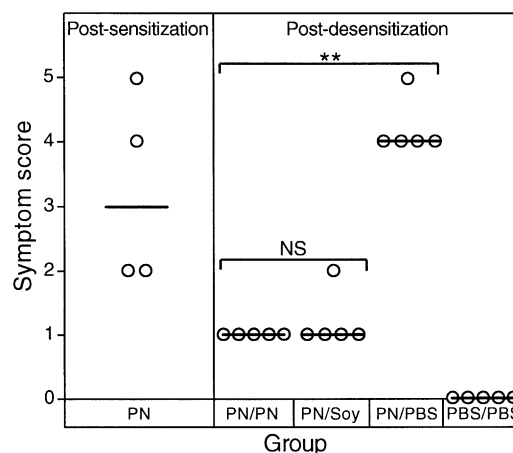


FIG 1. Symptom scores after peanut challenges of peanut-sensitized mice before and after desensitization. *PN*, Mice killed before undergoing IT; *PN/PN*, mice treated with crude peanut (0.5 mg of the highest dose); *PN/Soy*, crude soybean IT (1.0 mg of the highest dose); *PN/PBS*, placebo IT with PBS; *PBS/PBS*, naive mice sensitized/desensitized with PBS. Each circle corresponds to 1 animal, and the horizontal line represents the median of the group. Brackets indicate statistical differences between designated groups (** $P < .01$; NS, not significant).

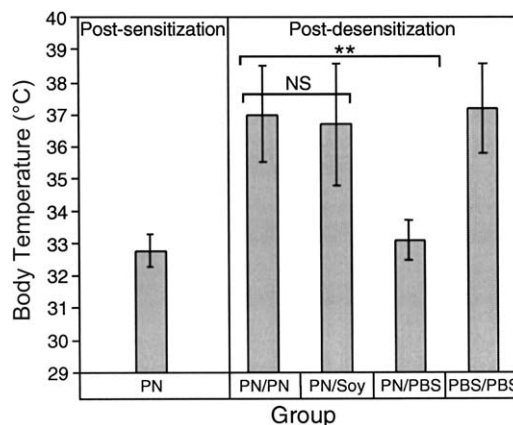


FIG 2. Core body temperatures after peanut challenges of peanut-sensitized mice before and after desensitization. *PN*, Mice killed before undergoing IT; *PN/PN*, mice treated with crude peanut (0.5 mg of the highest dose); *PN/Soy*, crude soybean IT (1.0 mg of the highest dose); *PN/PBS*, placebo IT with PBS; *PBS/PBS*, naive mice sensitized/desensitized with PBS. Values plotted are means $\pm$ SDs. Brackets indicate statistical differences between designated groups (** $P < .01$; NS, not significant).

peanuts at will but to provide patients some protection in case of accidental ingestion. Earlier experiments regarding desensitizing patients with subcutaneous injections of food extracts were poorly designed, and the results were not convincing.⁷ Likewise, early reports of oral desensitization were not well controlled.¹²

In 1992, Oppenheimer et al⁷ published their results from a trial of subcutaneous “rush” IT in patients allergic to peanuts. Given the partial rate of response in this study and others, the high rate of adverse reactions to traditional IT, and the frequent occurrence of accidental peanut

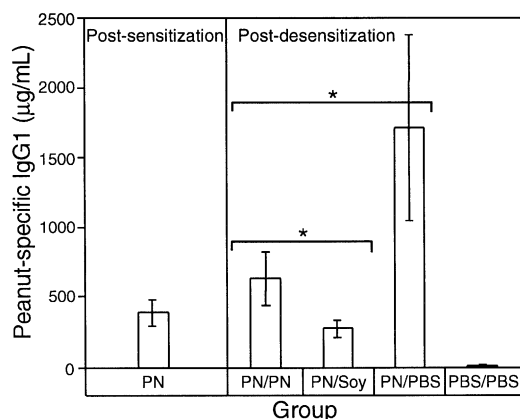


FIG 3. Peanut-specific IgG1 levels in the serum of peanut-sensitized mice before and after desensitization. *PN*, Mice killed before undergoing IT; *PN/PN*, mice treated with crude peanut (0.5 mg of the highest dose); *PN/Soy*, crude soybean IT (1.0 mg of the highest dose); *PN/PBS*, placebo IT with PBS; *PBS/PBS*, naive mice sensitized/desensitized with PBS. Values plotted are means $\pm$ SDs. Brackets indicate statistical differences between designated groups (* $P < .05$).

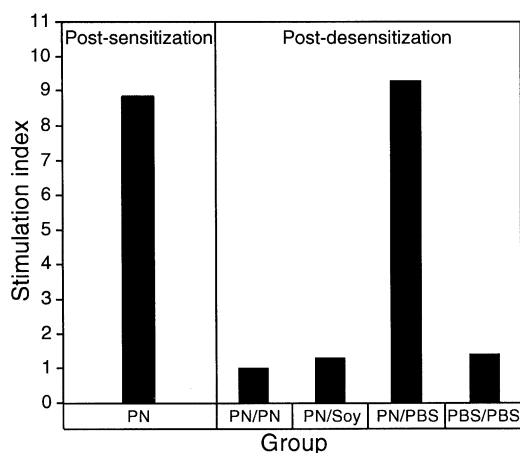


FIG 4. Splenocyte proliferative responses to peanut stimulation. Triplicate cultures of pooled spleen cells ($n = 5$) from nonchallenged mice of each group were stimulated with CPE 100 μ g/mL, medium alone, or PHA. After 3 days, the cells were pulsed for 6 hours with 1 μ Ci per well of [^{3} H]thymidine, harvested, and counted for β -radioactivity. Stimulation indexes were calculated as the mean of triplicates. *PN*, Mice killed before undergoing IT; *PN/PN*, mice treated with crude peanut (0.5 mg of the highest dose); *PN/Soy*, crude soybean IT (1.0 mg of the highest dose); *PN/PBS*, placebo IT with PBS; *PBS/PBS*, naive mice sensitized/desensitized with PBS. The average stimulation index with PHA for all the groups was $31\% \pm 5\%$.

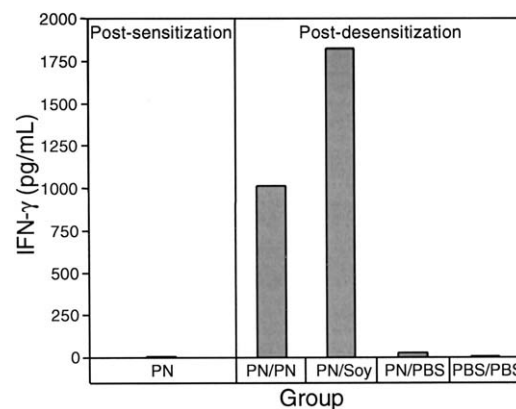


FIG 5. IFN- γ production by splenocytes stimulated with peanut extract. Pooled spleen cells (4×10^5 per well) from nonchallenged mice of each group ($n = 5$) were stimulated with CPE 100 μ g/mL. After 24 hours, the supernatants were collected, and IFN- γ levels were determined by flow cytometry with the Cytometric Bead Array method (BD PharMingen). Values plotted are means of triplicate determinations. *PN*, Mice killed before undergoing IT; *PN/PN*, mice treated with crude peanut (0.5 mg of the highest dose); *PN/Soy*, crude soybean IT (1.0 mg of the highest dose); *PN/PBS*, placebo IT with PBS; *PBS/PBS*, naive mice sensitized/desensitized with PBS.

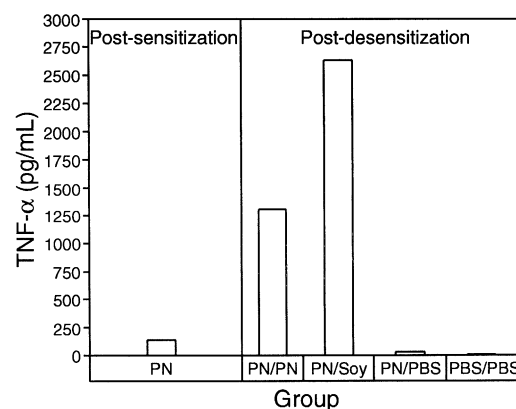


FIG 6. TNF- α production by splenocytes stimulated with peanut extract. Pooled spleen cells (4×10^5 per well) from nonchallenged mice of each group ($n = 5$) were stimulated with CPE 100 μ g/mL. After 24 hours, the supernatants were collected, and TNF- α levels were determined by flow cytometry with the Cytometric Bead Array method (BD PharMingen). Values plotted are means of triplicate determinations. *PN*, Mice killed before undergoing IT; *PN/PN*, mice treated with crude peanut (0.5 mg of the highest dose); *PN/Soy*, crude soybean IT (1.0 mg of the highest dose); *PN/PBS*, placebo IT with PBS; *PBS/PBS*, naive mice sensitized/desensitized with PBS.

ingestion, alternative forms of IT are necessary for this potentially fatal allergy. The sequence of events necessary to produce allergen-specific IgE suggests several potential avenues for immunomodulatory intervention. Novel immunotherapeutic strategies designed to alter the immune system's response to food allergens are being examined as potential treatment modalities for food allergy. These strategies include cytokine-modulated IT, immunostimu-

latory sequence-modulated IT, plasmid DNA IT, allergen-peptide IT, and engineered (mutated) allergen protein IT.¹³ All of these approaches strive to induce tolerance to specific food allergens. Unfortunately, all of these therapeutic options are several years away from becoming available to patients allergic to peanuts. Ongoing studies with the humanized, monoclonal anti-IgE antibody in patients allergic to peanuts are promising, but these studies

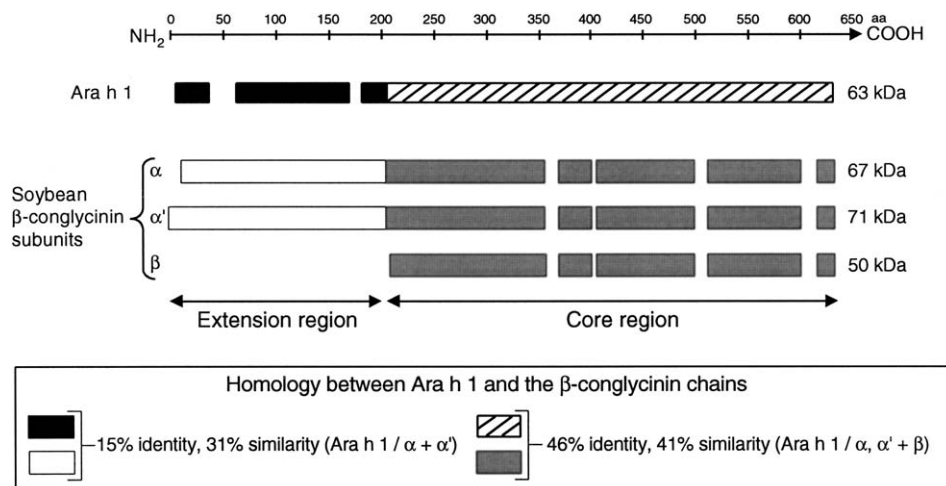


FIG 7. Schematic amino acid alignment of peanut allergen Ara h 1 and soybean β -conglycinin drawn from a multiple alignment of the full amino acid sequences by using CLUSTAL W 1.8.²² Interruptions in the bars indicate gaps introduced for the purpose of the alignment. Amino acid sequences represented by black (Ara h 1) and white (soybean) rectangles are 15% identical and 31% similar. Amino acid sequences represented by hatched (Ara h 1) and gray (soybean) rectangles are 46% identical and 41% similar. Percentages of identity and similarity for each region were calculated as the average of pairwise comparisons between Ara h 1 and the 3 β -conglycinin subunits.

are now only in phase I/II trials.¹⁴ If soybean IT for patients allergic to peanuts could exploit homologous proteins in soybeans, then the IT treatment might be available much sooner than engineered molecules.

The major seed storage proteins of legumes are globulins that are represented in most legumes by 2 different types of polypeptides: the nonglycosylated legumins and the glycosylated vicilins. All of the vicilin genes share a common ancestry.¹⁵ The major difference in the larger, glycosylated vicilin proteins is in the amino-terminal sequence (Fig 7). Similar information concerning the sequence homology exists for the conglutin and glycinin families of seed storage proteins. The sequence information for Ara h 1¹⁶ has shown that the gene for this peanut allergen is a part of a multigene family and that the allergen shares significant sequence homology with the vicilin storage proteins of other legumes (eg, soybean, pea, and common bean). The information generated demonstrating that the major peanut allergens are vicilin,¹⁶ conglutin,¹⁷ and glycinin¹⁸ proteins may explain why patients tend to have serum IgE to multiple legume proteins. Because the seed storage proteins have significant sequence homology among many plants, serum-specific IgE would bind to several vicilin, conglutin, and glycinin proteins from different species of legumes; however, the avidity and affinity of binding may be variable, suggesting sensitivity without clinical significance.

Extensive work has now been performed to identify the B- and T-cell epitopes on the peanut allergens.¹⁶⁻¹⁸ The primary immunodominant IgE-binding epitopes of Ara h 2 are in the amino-terminal half of the protein (Fig 8, A). The T-cell epitopes of the Ara h 2 peanut allergen are scattered throughout the entire amino acid sequence. It is

interesting to note that the T-cell epitopes of Ara h 2 in the carboxyl terminal are highly similar in amino acid sequence to its counterpart in soybean (Fig 8, B). This initial study in the peanut-allergic mouse model has shown that soy IT can be used to desensitize and/or downregulate the peanut-specific response in peanut-allergic mice. The symptom scores and the change in core body temperature after peanut challenge indicate that soy IT can change the clinical course in these mice in a way that is comparable to treatment with peanut IT. It is interesting to note that the IT-associated changes in peanut-specific immune response—ie, antibody levels and cytokines—indicate that the following immune response of soy IT is likely different from peanut IT in this model. For instance, the changes in peanut-specific antibody levels underlying soy IT are distinctly different from those obtained after peanut IT. The changes in peanut-specific cellular proliferation, as well as the cytokine profile after soy IT, are similar to those observed after peanut IT. The changes in peanut-induced IFN- γ and TNF- α seem to indicate that a shift to a T_H1 lymphocyte response occurs after both types of IT, with the shift in soy IT possibly being more robust. Successful specific IT is characterized by the induction of peripheral tolerance to the injected allergen(s),^{19,20} which is demonstrated by diminished lymphoproliferative responses to the allergen(s) and an immunodeviation of the remaining T-cell reactivity toward a more T_H1/0-like response associated with the production of higher amounts of IFN- γ .¹⁹⁻²¹ A limitation of this study is that the IT was not performed in an orally sensitized model of food allergy. Additionally, extrapolating the results of this study to peanut-sensitized humans may be problematic because most patients allergic to peanuts can eat soybeans and therefore already have an immune response to soy.

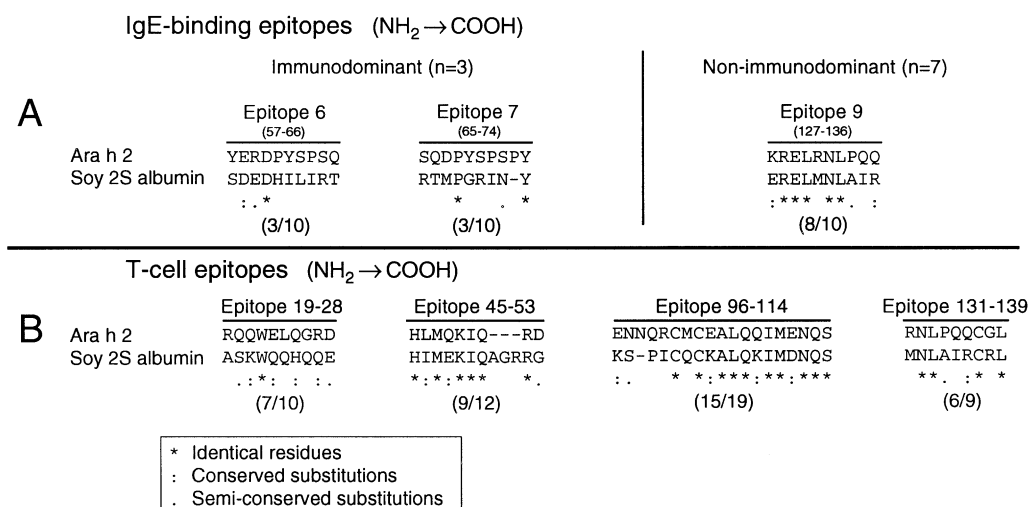


FIG 8. Amino acid sequence alignment of peanut allergen Ara h 2 and soybean 2S albumin. **(A)** There are 10 IgE-binding epitopes on Ara h 2. Three of the 10 epitopes are immunodominant (numbered 2, 6, and 7). Immunodominant epitopes 6 and 7 are shown. There are only 3 of 10 identical or similar amino acids between Ara h 2 and the soybean 2S albumin (GenBank AAD09630) in these 2 epitopes. In the nonimmunodominant epitopes (epitope 9, representative example), there is a high degree of identity or similarity (8/10). **(B)** There are 4 T-cell epitopes on Ara h 2. These epitopes have 67% to 79% amino acid identity or similarity with the aligned soybean 2S albumin sequence. The pairwise alignment was performed on the full amino acid sequences by using CLUSTAL W 1.8 and trimmed to focus on Ara h 2–different epitopes.

Taken together, our preliminary data of soy IT in a murine model clearly underscore the feasibility of our approach. Delineation of the molecular mechanisms responsible for the immunologic shift to a T_H1-type response and decreased anaphylactogenic activity by using a soy IT model will likely provide new therapeutic interventions for peanut allergy. We plan to use our extensive knowledge of the allergens involved in peanut hypersensitivity to gain a better understanding of the human immune response involved in this hypersensitivity reaction and the potential immunotherapeutic capabilities of different allergens. We will continue to study the innovative idea that soybean IT, because of the significant amino acid sequence homology in the legume seed storage proteins, will desensitize patients with peanut hypersensitivity reactions.

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