

Mutational analysis of the IgE-binding epitopes of P34/Gly m Bd 30K

Ricki M. Helm, PhD,^a Gael Cockrell, BS,^a Cathie Connaughton, BS,^a C. Michael West, BS,^b Eliot Herman, PhD,^c Hugh A. Sampson, MD,^d Gary A. Bannon, PhD,^b and A. Wesley Burks, MD^a Little Rock, Ark, Beltsville, Md, and New York, NY

Background: Peanuts and soybeans are 2 foods that have been shown to be responsible for many atopic disorders. Because of their nutritional benefit, soybean proteins are now being used increasingly in a number of food products. Previous studies have documented multiple allergens in soybean extracts, including glycinin, β -conglycinin, and the P34/Gly m Bd 30K protein.

Objective: Our overall goal was to identify soybean-specific allergens to begin to understand molecular and immunochemical characteristics of legume proteins. The specific aim of the current investigation was to identify the essential amino acid residues necessary for IgE binding in the 5 distinct immunodominant epitopes of P34/Gly m Bd 30K.

Methods: Serum IgE from 6 clinically sensitive soybean-allergic individuals was used to identify P34/Gly m Bd 30K in the native and single amino acid substituted peptides with use of the SPOTS peptide synthesis technique to determine critical amino acids required for IgE binding.

Results: The intensity of IgE binding and epitope recognition by serum IgE from the individuals varied substantially. With use of serum from 6 clinically soybean-sensitive individuals, 2 of the 5 immunodominant epitopes could be mutagenized to non-IgE binding peptides.

Conclusions: Single-site amino acid substitution of the 5 immunodominant epitopes of Gly m Bd 30K with alanine revealed that IgE binding could be reduced or eliminated in epitopes 6 and 16 in the serum obtained from 6 soybean-sensitive patients. (*J Allergy Clin Immunol* 2000;105:378-84.)

Key words: Soybean allergen, P34/Gly m Bd 30K, B-cell epitopes

Soybeans and peanuts are both members of the legume plant family and they share common antigenic fractions. Patients allergic to one member often have IgE antibodies that react to other members of the legume family. Although cross-reacting proteins among soybeans and

Abbreviations used

DBPCFC:	Double-blind, placebo-controlled food challenge
Gly m Bd:	Glycine max band
rP34:	Recombinant P34 protein
TBST:	TRIS-buffered saline solution with 0.05% Tween 20

peanuts are immunochemically frequent, clinical responses to more than one is not common.¹⁻³ A comparison of IgE immunoblots to soybean extracts with common legumes showed that soybean extracts shared several common as well as unique proteins with other members of the legume family.^{4,5}

Previous studies have documented multiple allergens in soybean extracts, including glycinin, β -conglycinin, and the Glycine max band (Gly m Bd) 30K protein.⁶⁻¹¹ Ogawa et al^{7,8} initially described a soybean band designated Gly m Bd 30K as a 7S soybean protein allergen, assigning it as the major allergenic protein in soybean extracts that bound specific IgE in the serum of 65% of patients with atopic dermatitis in Japan. The first 15 amino acids of Gly m Bd 30K were identical to those of a 34-kd soybean seed storage vacuole protein identified by Kalinski et al.^{9,10} P34 is an outlying member of the papain superfamily^{9,10} that appears to be a *Pseudomonas* defense protein.¹¹

The most efficient way to avoid soybean allergy is strict avoidance of food products containing allergenic proteins. Several methods are being conducted to reduce the allergenicity of allergens, including enzymatic methods, physical treatment, or development of soybean seeds that lack the allergenic protein.¹² A combination of both genetically selected soybean cultivars and physicochemical separation methods appears to be the most promising method. With this in mind, our goal was to identify soybean allergens and specifically determine the presence of unique soybean protein epitopes in their amino acid structure that could potentially be mutagenized to non-IgE-binding proteins. The prevalence of the hypersensitivity reactions to the allergen identified as Gly m Bd 30K and its homology to the previously identified protein P34, a constituent of protein storage vacuoles in soybean seed cotyledons, made this protein an ideal candidate for identification and characterization of the major IgE-binding epitopes.¹³ The specific aim of the current investigation was to identify the essential amino

From the ^aDepartment of Pediatrics, University of Arkansas for Medical Sciences, Arkansas Children's Nutrition Center, Little Rock, Ark, the ^bDepartment of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, the ^cClimate Stress Laboratory, United States Department of Agriculture, Agriculture Research Service, Beltsville, Md, and the ^dDivision of Pediatric Allergy and Immunology, Mt Sinai Medical School, New York, NY.

Supported by a grant from the Arkansas Children's Nutrition Center (No. USDA-ARS 0524-0022*) and grant No. AI-24439 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.^d

Received for publication July 6, 1999; revised Oct 6, 1999; accepted for publication Oct 13, 1999.

Reprint requests: Ricki M. Helm, PhD, Department of Pediatrics, University of Arkansas for Medical Sciences, Arkansas Children's Nutrition Center 1120 Marshall St, Little Rock, AR 72202.

1/1/103611

acid residues necessary for IgE binding in the 5 distinct immunodominant epitopes in this major soybean allergen. In this article we report the localization of specific amino acids that can be substituted to render immunodominant epitopes 6 and 16 to non-IgE-binding peptides.

MATERIAL AND METHODS

Serum IgE

Twelve patients with atopic dermatitis and a positive immediate skin test response to soybean extracts were confirmed to be sensitive to soybean protein by double-blind, placebo-controlled food challenge (DBPCFC).² The individual sera were diluted 1:20 (vol/vol) in TRIS-buffered saline solution (25 mmol/L TRIS–hydrochloric acid, pH 7.2, 150 mmol/L sodium chloride) with 0.05% Tween 20 (TBST) for immunoblot analysis and in 1:5 (vol/vol) TBST with blocking solution as recommended by the manufacturer (Genosys Biotechnologies, The Woodlands, Tex) for mapping of IgE-binding regions and epitopes of the Gly m Bd 30K allergen. The Human Advisory Committee approved all studies at the University of Arkansas for Medical Sciences and Mt Sinai School of Medicine.

Soybean extracts

Preparations of defatted soybean flakes were obtained from commercial sources (Dr Jim Brooks, Ross Laboratories, Columbus, Ohio). Defatted (Soxhlet extraction with petroleum ether) soybean flakes were ground to a fine powder in a mortar and pestle and extracted (1:20 wt/vol) in 20 mmol of sodium phosphate, 1 mmol of sodium chloride, pH 7.2, overnight at 4°C.

A clone designated P34,⁹ representing the protein seed storage vacuole protein, was used as a source of recombinant protein. PCR techniques were applied to isolate the protein-coding region of this clone. This portion was then subcloned into the pet-24(b)+ vector for expression of recombinant protein in *Escherichia coli*. The protein was expressed with a 6x-his tag on its carboxyl terminus. The 6x-his tag allowed the purification of full-length protein by nickel resin column affinity chromatography. The purified protein was characterized by immunoblot analysis with serum IgE from soybean-sensitive individuals. Results indicate that the recombinant P34 protein was recognized with a similar IgE-binding intensity as the native protein, suggesting that the recombinant protein can be used for future studies. The protein was desalted into PBS, concentrated (4.2 mg/mL), filter sterilized, and stored for future use.

SDS-PAGE/Western immunoblot analysis

SDS-PAGE was performed according to the method of Laemmli.¹⁴ All gels were composed of 12.5% acrylamide resolving gel and 4% acrylamide stacking gel. Electrophoretic transfer of proteins and immunoblotting were performed according to standard procedures.¹⁵ The blots were blocked with TBST with 1% BSA 60 minutes before incubation with antibodies diluted in TRIS-buffered saline buffer for at least 12 hours at 4°C or for 4 hours at room temperature. Primary IgE antibody was detected with radiolabeled anti-IgE antibody (Sanofi Pasteur Institute, Chaska, Minn) diluted 1:5 (vol/vol) in TRIS-buffered saline buffer. Blots were washed 3 times in buffer, air dried, and exposed to Kodak X-Omat x-ray film at –70°C for 18 to 144 hours and developed in an automatic developer (Konica SRX-101).

IgE binding of mutagenized immunodominant epitopes

The immunodominant epitopes of P34 recognized by 4 clinically soybean-sensitive individuals¹³ were used to generate individual peptides for IgE-binding studies. Substitution of an alanine residue

for each of the wild-type amino acids in the sequence was used to determine which amino acids were essential for IgE binding. Individual peptides were synthesized on a cellulose membrane containing free hydroxyl groups with use of Fmoc amino acids according to the manufacturer's instructions (Genosys Biotechnologies). Synthesis of each peptide was started by esterification of an Fmoc amino acid to the cellulose membrane. After washing, all residual amino functions on the sheet were blocked by acetylation to render the cellulose membrane unreactive during subsequent steps. Each additional Fmoc amino acid was esterified to the previous one by this same process. After addition of the last amino acid in the peptide chain, the amino acid side chains were deprotected with a mixture of dichloromethane–trifluoroacetic acid–tri-isobutylsilane (1:1:0.05, vol/vol), followed by treatment with dichloromethane and washing with methanol. Membranes containing synthesized peptides were washed with TRIS-buffered saline solution and then incubated with the Genosys blocking solution overnight at room temperature. The membranes were probed immediately with serum IgE as described above or stored at –20°C until used. IgE binding was determined by incubating cellulose membranes containing the synthesized peptides with serum from individuals with documented soybean hypersensitivity and, as control, serum from an individual with high-titer IgE with no known food allergy. Primary antibody was detected with radiolabeled anti-IgE as described for autoradiographs of SDS-PAGE/Western IgE immunoblot analysis.

RESULTS

SDS-PAGE of soybean extracts and recombinant P34

Fig 1 shows a representative profile of an immunoblot of IgE binding to a crude soybean extract and to recombinant P34 (rP34) (lanes 2 and 4, respectively). The IgE-binding is most intense at the 30-kD rP34 protein band. The IgE immunoblot sensitivity reveals radiographic binding at both higher and lower molecular weight bands that are not discernible in the Coomassie blue-stained photograph. These bands are evident in the original stained gel and represent complexes and breakdown products of the rP34 molecule common to many recombinant proteins isolated under these conditions. Control serum from nonleguminous-sensitive patients did not bind to any of the bands from our rP34 material nor did serum IgE from soybean-sensitive individuals bind to extracts of *E coli* in these regions (data not shown).

IgE binding to immunodominant epitopes

The prevalence of serum IgE binding to epitopes 1, 6, 13, 15, and 16 were previously identified as immunodominant epitopes with use of serum from 4 clinically soybean-sensitive individuals.¹³ In the current investigation we were able to add data from an additional 8 soybean-sensitive individuals. To determine the extent of patients' serum IgE binding to the immunodominant epitopes, each epitope was synthesized as a single spot on SPOTs membranes and incubated with serum from individual patients. The immunodominant epitopes are listed in Fig 2 with a representative blot of 6 patients' serum IgE-binding pattern. The intensity of IgE binding and epitope recognition by serum IgE from the individuals varied substantially. For example, serum IgE from

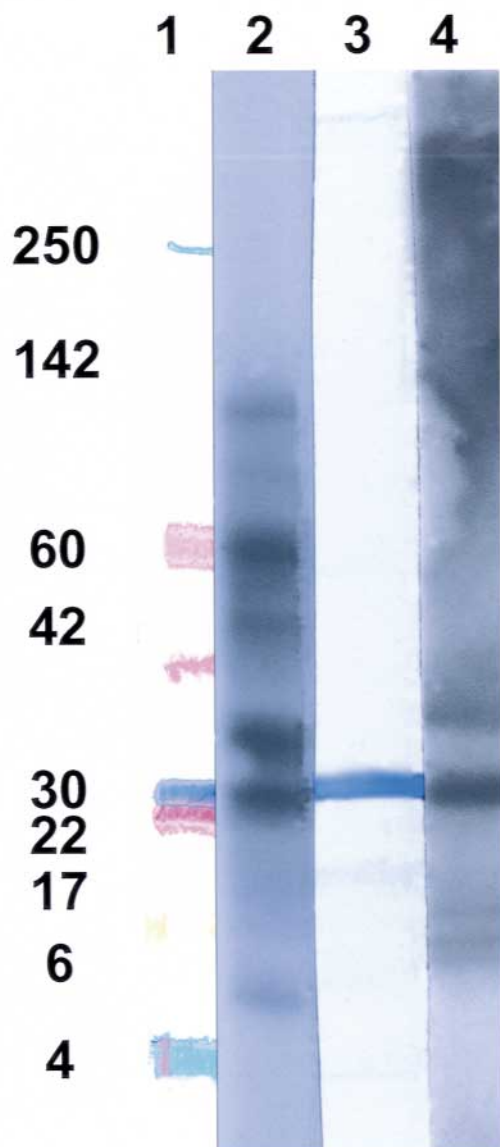


FIG 1. SDS-PAGE IgE immunoblot analysis of crude soybean extract and rP34. *Lane 1*, Molecular weight markers. *Lane 2*, IgE immunoblot autoradiograph of crude soybean extract. *Lane 3*, Coomassie blue-stained rP34 protein. *Lane 4*, IgE immunoblot autoradiograph of rP34 protein.

patients 5 and 3 bound all 5 epitopes with the same intensity. Patients 7, 8, and 1 demonstrated more intense staining to epitope 15, whereas serum from patient 3 recognized epitope 13 as the predominant epitope. This difference in intensity and binding was characteristic of serum IgE from all 12 patients tested. Because the intensity of the binding varied, we chose to test patient serum against the 5 immunodominant epitopes. Table I shows a summary of the 12 patients' serum IgE that were tested for binding to the 5 immunodominant epitopes. We had 2 patients who did not recognize any of the 5 immunodominant epitopes and 5 who recognized all the epitopes, and the remaining bound from 2 to 4 of the 5

TABLE I. Summary of P34 immunodominant epitope mapping by serum IgE from soybean-sensitive individuals

	P34 epitope binding				
	1	6	13	15	16
1*	+	±	+	+	+
2	+	+	±	+	±
3*	+	±	+	+	+
4	+	+	—	+	+
5*	+	+	+	+	+
6*	+	±	+	+	+
7*	+	+	+	+	+
8*	+	±	±	+	+
9	—	+	+	—	+
10	—	—	—	—	—
11	—	—	—	—	—
12	—	+	+	—	+

+, Positive binding; ±, marginal binding; —, negative binding.

*Selected for mutagenized epitope mapping.

TABLE II. Mutagenesis of P34/Gly m Bd 30K epitope 1

SPOT No.	Mutagenized epitope 1	Position change
1	ALVLLLFSL	F1A
2	FAVLLLFSL	L2A
3	FLALLLFSL	V3A
4	FLVALLFSL	L4A
5	FLVLALFSL	L5A
6	FLVLLAFSL	L6A
7	FLVLLLFSL	F7A
8	FLVLLLFSL	S8A
9	FLVLLLFSL	L9A
10	FLVLLLFSLA	L10A
11	FLVLLLFSL	Native

Note: If an alanine residue resided in mutagenized location, glycine was used as substituted amino acid.

immunodominant epitopes. This is in agreement with our initial results in that there appears to be a great amount of diversity in the immunodominant IgE-binding epitopes.

IgE binding to mutagenized immunodominant epitopes

To determine the amino acids that are critical to IgE binding of the 5 immunodominant epitopes, we mutagenized the amino acid sequences by substituting alanine at each position and compared the IgE binding to native (wild-type) epitope. Table II depicts the position change for epitope 1. Each epitope was modified in this manner in a series of replicates for analysis with IgE from 6 of the P34-sensitive individuals. Fig 3 shows the results from 3 of these individuals.

Serum IgE from patient 12 recognized only epitopes 6, 13, and 16. Alanine substitutions in epitope 6 at positions 6 and 7 and at position 7 in epitope 16 showed reduced IgE binding. Alanine substitutions at positions 1-6 and 8 showed increased and position 7 reduced IgE binding with this patient's serum IgE to epitope 16. Epitope 13

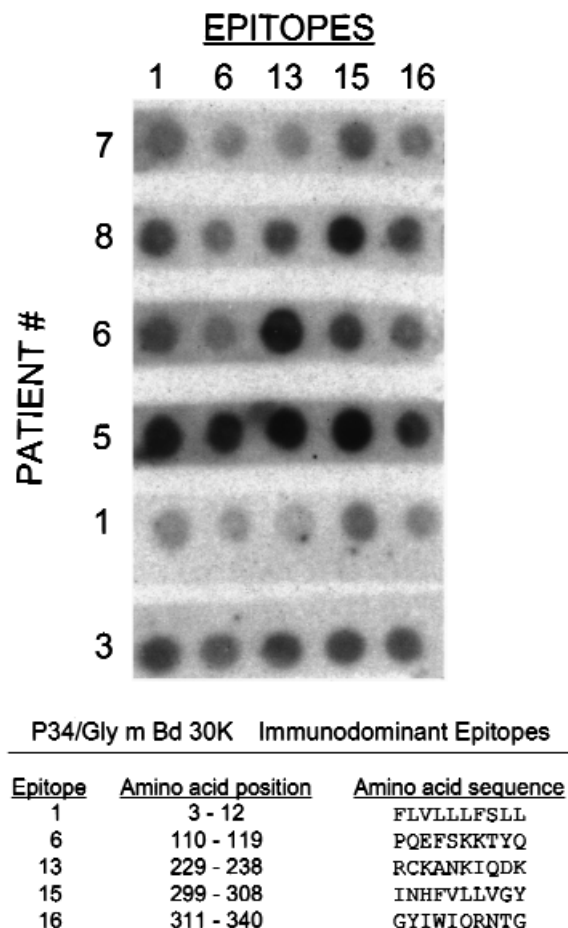


FIG 2. Autoradiograph of patient serum IgE binding to 5 immunodominant P34/Gly m Bd 30K epitopes.

could not be mutagenized to a non-IgE-binding peptide with alanine substitutions at any position in the peptide with serum from patient 12.

Serum IgE from patients 8 and 3 recognized all 5 epitopes. Consistent with patient 12, serum from both patients failed to bind IgE at positions 6 and 7 of epitope 6. Serum from patient 8 demonstrated that an alanine substitution did not significantly alter the IgE binding in the remaining sites of the epitopes investigated. The autoradiograph for patient 3, in addition to reduced IgE binding at position 6 and 7 of epitope 6, revealed both increased and decreased levels of radiostained intensity in the remaining sites of the immunodominant epitopes.

Table III summarizes the extent of IgE binding to mutagenized epitopes with the 6 individuals we tested. Notable in this table is the consistency with which we could or could not mutagenize IgE binding by alanine substitution. Epitopes 1, 13, and 15 could not be mutagenized to a non-IgE-binding peptide, whereas epitope 6 could be altered to a non-IgE-binding peptide by an alanine substitution at positions 6 and 7 in the amino acid sequence. Epitope 16 was more variable and could be mutagenized to a non-IgE-binding peptide at position 7

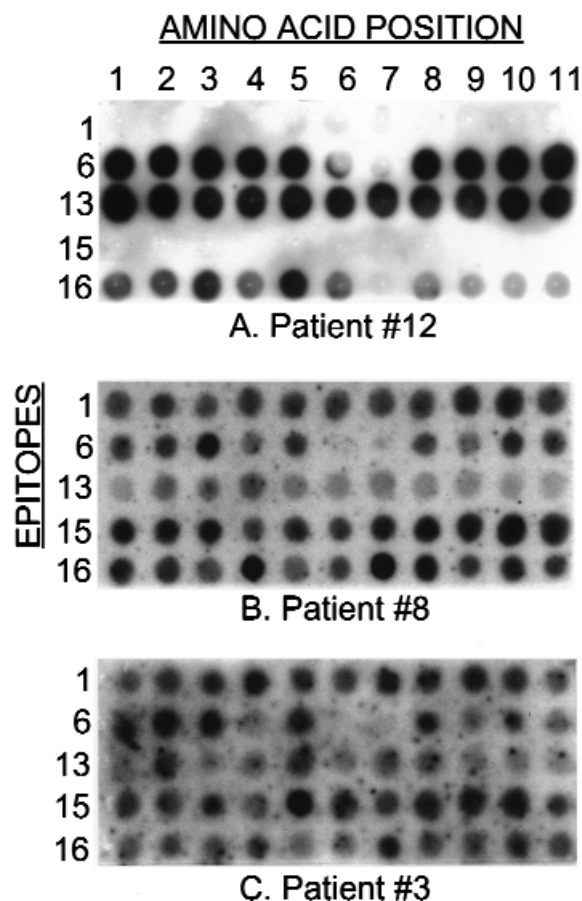


FIG 3. Autoradiograph of representative patient serum IgE binding to mutagenized P34/Gly m Bd 30K epitopes.

for 4 patients (patients 4, 5, 7, and 12) and at positions 5 and 6 for 2 patients (patients 3 and 8). This suggested that we could mutagenize epitopes 6 and 16 to non-IgE-binding peptides by substituting alanine at specific sites in the sequence of rP34 for patients 3, 8, and 12.

DISCUSSION

Five immunodominant IgE-binding epitopes were previously identified with use of serum IgE from 4 soybean-sensitive individuals by B-cell epitope mapping with overlapping peptides for the soybean protein identified as Gly m Bd 30K (Gly m Bd30K/P34), a plant thiol protease.¹³ This protein has been identified as the major soybean allergen with a frequency of 65% in patients with atopic dermatitis with food allergy.⁸ In this study we report the results of mutagenizing the 5-immunodominant epitopes on the P34/Gly m Bd 30K soybean for the identification of amino acids critical for IgE binding. The single-site amino acid substitution of the 5 epitopes revealed that IgE binding could be reduced or eliminated depending on the serum of individual patients.

Adverse reactions to various food products are a com-

TABLE III. Summary of 6 patients' sera used to map mutagenized immunodominant epitopes

Epitope	Amino acid position (alanine)										Weight
	1	2	3	4	5	6	7	8	9	10	
Patient 12											
1	—	—	—	—	—	—	—	—	—	—	—
6	+	+	+	+	+	—	—	+	+	+	+
13	+	+	+	+	+	+	+	+	+	+	+
15	—	—	—	—	—	—	—	—	—	—	—
16	+	+	+	+	+	+	—	+	+	+	+
Patient 9											
1	—	—	—	—	—	—	—	—	—	—	—
6	+	+	+	+	+	—	—	+	+	+	+
13	+	+	+	+	+	+	+	+	+	+	+
15	—	—	—	—	—	—	—	—	—	—	—
16	+	+	+	+	+	+	—	+	+	+	+
Patient 4											
1	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	—	—	+	+	+	+
13	—	—	—	—	—	—	—	—	—	—	—
15	+	+	+	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	—	+	+	+	+
Patient 3											
1	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	—	—	+	+	+	+
13	+	+	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+	+	+	+
16	+	+	+	+	—	—	+	+	+	+	+
Patient 5											
1	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	—	—	+	+	+	+
13	+	+	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	—	+	+	+	+
Patient 8											
1	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	—	—	+	+	+	+
13	+	+	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+	+	+	+
16	+	+	+	+	—	—	+	+	+	+	+

mon problem faced by primary care physicians. Among the numerous reactions ascribed to soy products, hypersensitivity, in contrast to enterocolitis, is described as an abnormal immunologic response in which IgE is the principal immunoglobulin. In most studies the diagnosis of soy allergy has been based on clinical evaluation or case histories reported by parents without substantial scientific diagnostic criteria. In a recent study 1% of children with allergic disease had documented soy allergy and all of them had atopic dermatitis.¹⁶ In related studies with a 3- to 5-year follow-up, the frequency of soy reactions was almost nonexistent,¹⁷ with other studies proclaiming a more transient adverse soy reaction.^{18,19} The reports suggest a wide discrepancy in soy reactions, ranging from nonexistent to transient and documented soy reactions. This discrepancy is manifested in patient selection criteria and the method used to document soy reactions. Our study involved patients with soy reactions clinically proved by DBPCFC. After diagnosis of a food allergy, the only

proved therapy is elimination of the offending allergen.²⁰ For those individuals with soybean allergy a more direct approach to treatment of this food allergy is desirable.

Although the incidence of allergy to soybean proteins is quite low in comparison with other major food proteins, the gradually increasing consumption of soybean products makes the identification and characterization of major soy allergens a focus for investigation. Herian et al⁵ identified 3 different patterns of immunoblots in adult patients with soybean allergy on the basis of molecular weights. Serum bound IgE patterns were resolved into those individuals who had IgE-recognizing proteins with molecular weights of 50 to 60 kd, 20 kd, or 14 kd. Awazu et al²¹ identified 5 proteins with molecular weights ranging from 20 to 78 kd in the whey and globulin fraction of soybean extracts. None of these proteins had an IgE-binding frequency greater than 50%. In studies with 8 serum samples from soy protein-allergic children, immunoblots to crude soy proteins demonstrat-

ed that IgE- and IgG-specific antibodies to crude soy extracts were elevated in patients with positive DBPCFC, but none of the soy fractions investigated were clearly more antigenic.⁶ In a study carried out in Japan, SDS-PAGE and immunoblotting analyses revealed that 19% of patients with atopic dermatitis possessed IgE antibodies specific for soybean proteins.⁸ At least 16 proteins were recognized, with detection frequencies ranging from 1.4% to 2.9% for 2S and 11S components to as high as 65% for the 7S-globulin fraction that contains Gly m Bd 30K. The remaining protein components had IgE binding frequencies of less than 30%. The variability in the number of epitopes recognized reflects both the polyclonal nature of the immune response and the subject variability in epitope recognition.

The development of methods to reduce the allergenicity of soybean proteins is required because no other food material is used in as many foods as soybean throughout the world. Hidden soybean allergens in processed foods have been shown to provoke severe reactions in sensitive individuals.²² A combination of both genetically selected soybean cultivar mutants lacking the α - and α' -subunits of conglycinin and physicochemical separation methods to remove Gly m Bd 30K from soy milk has been described.¹² Although this is a very simple method, efficient industrial application procedures have not been developed to produce a nonallergenic or very-low-allergenic soybean protein source.

Another method to reduce the allergenicity of soybean proteins would be to introduce genes that would produce hypoallergenic versions of the allergens.²³⁻²⁵ The intensity of binding to wild-type peanut allergen was routinely high, whereas binding to mutated epitopes was markedly decreased at specific sites in each of the peanut epitopes. In contrast, the IgE binding to wild-type P34/Gly m Bd 30K epitopes did not show the same degree of intense binding as evidenced by autoradiographic analysis, nor could we mutate each of the epitopes to non-IgE-binding peptides. Peanut sensitivity, which is a life-long, life-threatening hypersensitivity, is a more severe food allergy leading to anaphylactic episodes. Soybean hypersensitivity, which is usually outgrown by age 5 years or less, is associated more with gastrointestinal symptoms and normally does not lead to an anaphylactic episode.

The elucidation of the IgE-binding epitopes on P34/Gly m Bd 30K, the major allergen of soybean in atopic dermatitis identified by Ogawa et al⁸ and the determination of amino acids essential to IgE binding could provide the information necessary to alter the gene responsible for encoding a non-IgE-binding protein. Although other proteins are involved in soybean hypersensitivity, we chose the P34 molecule as our first soybean allergen to mutagenize and to test for insertion into the soybean genome. This approach will require that we suppress the native gene and determine that our altered gene product will retain its native function and properties in the soybean plant and seed. We propose to test our hypothesis with use of in vitro transformation techniques

to determine the potential of producing a soybean product with reduced P34 serum IgE binding. Our overall approach is to prepare moderately modified proteins by site-directed amino acid substitutions of soybean allergens and use of cosuppression of native allergens to produce a hypoallergenic soybean plant.

REFERENCES

1. Sampson HA. Immunopathogenic role of food hypersensitivity in atopic dermatitis. *Acta Derm Venereol* 1992;176:34-7.
2. Burks AW, Mallory SB, Williams LW, Shirrell MA. Atopic dermatitis: clinical relevance of food hypersensitivity reactions. *J Pediatr* 1988;113:447-51.
3. Bernhisel-Broadbent J, Sampson HA. Cross allergenicity in the legume botanical family in children with food hypersensitivity. *J Allergy Clin Immunol* 1989;83:435-40.
4. Burks AW, Williams LW, Helm RM, Connaughton C, Cockrell G, O'Brien T. Identification of a major peanut allergen Ara h I, in patients with atopic dermatitis and positive peanut challenge. *J Allergy Clin Immunol* 1991;88:172-9.
5. Herian AM, Taylor SL, Bush RK. Identification of soybean allergens by immunoblotting with sera from soy-allergic adults. *Int Arch Allergy Appl Immunol* 1990;92:193-8.
6. Burks AW, Brooks JR, Sampson HA. Allergenicity of major component proteins of soybean determined by enzyme-linked immunosorbent assay (ELISA) and immunoblotting in children with atopic dermatitis and positive soy challenges. *J Allergy Clin Immunol* 1988;81:1135-42.
7. Ogawa T, Bando N, Tsuji H, Okijima H, Nishikawa L, Sasoka K. Investigations of the IgE-binding proteins in soybeans by immunoblotting with sera from soybean-sensitive patients with atopic dermatitis. *J Nutr Sci Vitamin* 1991;37:555-65.
8. Ogawa R, Tsuji H, Bando N, Kitamura K, Zhu YL, Hirano H, et al. Identification of the soybean allergenic protein, Gly m Bd 30k, with the soybean seed 34-kDa oil-body-associated protein. *Biosci Biotech Biochem* 1993;57:1030-3.
9. Kalinski A, Weisemann JM, Matthews BF, Herman EM. Molecular cloning of a protein associated with soybean seed oil bodies that is similar to thiol proteases of the papain family. *J Biol Chem* 1990;265:13843-48.
10. Kalinski A, Melroy DL, Dwivedi RS, Herman EM. A soybean vacuolar protein (P34) related to thiol proteases is synthesized as a glycoprotein precursor during seed maturation. *J Biol Chem* 1992;267:12068-76.
11. Ji C, Boyd C, Slaymaker D, Okinaka U, Herman EM, Keen NT. Purification and characterization of a 34 kDa syringolide binding protein from soybean. *Proc Natl Acad Sci U S A* 1998;95:3306-11.
12. Samoto M, Takahashi K, Fukuda Y, Nakamura S, Kawamura Y. Substantially complete removal of the 34 kDa allergenic soybean protein, Gly m Bd 30K from soy milk of a mutant lacking the α and α' -subunits of conglycinin. *Biosci Biotech Biochem* 1996;60:1911-3.
13. Helm RM, Cockrell G, Herman E, Burks AW, Sampson HA, Bannon GA. Cellular and molecular characterization of a major soybean allergen. *Int Arch Allergy Immunol* 1998;117:29-37.
14. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-5.
15. Towbin H, Staehlin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc Natl Acad Sci U S A* 1979;76:4350-4.
16. Bruno G, Giampietro PG, Del Guercio MJ, Gallia P, Giovannini L, Lovati C, et al. Soy allergy is not common in atopic children: a multicenter study. *Pediatr Allergy Immunol* 1997;8:190-3.
17. Cantani A, Lucenti P. Natural history of soy allergy and/or intolerance in children, and clinical use of soy-protein formulas. *Pediatr Allergy Immunol* 1997;8:59-74.
18. Hattevig G, Kjellman B, Johansson SGO, Bjorksten B. Clinical symptoms and IgE responses to common food proteins in atopic and healthy children. *Clin Allergy* 1984;14:551-5.
19. Host A, Halken S. A prospective study of cow milk allergy in Danish infants during the first three years of life. *Allergy* 1990;45:587-96.
20. Sampson HA. Adverse reactions to foods. In: Middleton E Jr, Reed CE,

- Ellis EF, Adkinson NF Jr, Yunginger JW, Busse WW, editors. Allergy, principles and practice. St Louis: Mosby-Year Book; 1993. p 1661-86.
21. Awazuwara H, Kawai H, Maruchi N. Major allergens in soybean and clinical significance of IgG4 antibodies investigated by IgE- and IgG4-immunoblotting with sera from soybean-sensitive patients. *Clin Exp Allergy* 1997;27:325-32.
 22. Vidal C, Perez-Carral C, Chomon B. Unsuspected sources of soybean exposure. *Ann Allergy Asthma Immunol* 1997;79:350-2.
 23. Burks AW, Shin D, Cockrell G, Stanley JS, Helm RM, Bannon GA. Mapping and mutational analysis of the IgE-binding epitopes on Ara h 1, a legume vicilin protein and a major allergen in peanut hypersensitivity. *Eur J Biochem* 1997;245:334-9.
 24. Stanley JS, King N, Burks AW, Huang SK, Sampson HA, Cockrell G, et al. Identification and mutational analysis of the immunodominant IgE binding epitopes of the major peanut allergen Ara h 2. *Arch Biochem Biophys* 1997;343:244-53.
 25. Rabjohn P, Helm EM, Stanley JS, West CM, Sampson HA, Burks AW, et al. Molecular cloning and epitope analysis of the peanut allergen, Ara h 3. *J Clin Invest* 1999;103:535-42.