

Assessing the Allergenicity of Proteins Introduced into Genetically Modified Crops Using Specific Human IgE Assays

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Global commercial production of genetically modified (GM) crops has grown to over 67 million hectares annually, primarily of herbicide-tolerant and insect protection crop varieties. GM crops are produced by the insertion of specific genes that either encode a protein, or a regulatory RNA sequence. A comprehensive safety evaluation is conducted for each new commercial GM crop, including an assessment of the potential allergenicity of any newly introduced protein. If the gene was derived from an allergenic organism, or the protein sequence is highly similar to a known allergen, immunoassays, e.g., Western blot assays and enzyme-linked immunosorbent assay tests, are performed to identify protein-specific IgE binding by sera of individuals allergic to the gene source, or the source of the sequence-matched allergen. Although such assays are commonly used to identify previously unknown allergens, criteria have not been established to demonstrate that a protein is unlikely to cause allergic reactions. This review discusses factors that affect the predictive value of these tests, including clinical selection criteria for serum donors, selection of blocking reagents to reduce nonspecific antibody binding, inhibition assays to verify specificity of binding, and scientifically justified limits of detection (sensitivity) in the absence of information regarding biological thresholds.

Genetically modified (GM) plants are produced by the insertion of specific genes (DNA sequences), which encode proteins, or genes that express anti-sense RNA transcripts that inhibit the production of specific plant or viral proteins. Calgene developed the first GM food crop, the delayed-ripening FLAVR SAVRTM tomato, by inserting an antisense gene for the enzyme polygalacturonase, thereby delaying fruit softening (1). In 1996, Monsanto's herbicide-

tolerant soybeans were commercialized. The soybeans express a bacterial homolog of a plant enzyme required for aromatic amino acid biosynthesis. Since then, a number of insect protection, disease-resistant, herbicide-tolerant, and nutritionally enhanced crop varieties of more than 20 agriculturally important crops have been developed. The global commercial production of all GM crops has grown to more than 67 million hectares annually (1–3). The GM plants and/or the food and feed produced from those plants are regulated by various governmental agencies to ensure safety to the consumer and the environment (4). In the United States, 3 agencies share responsibility for oversight of crops produced through biotechnology: the U.S. Department of Agriculture (USDA), the U.S. Food and Drug Administration (FDA), and the U.S. Environmental Protection Agency (EPA). Although the primary responsibilities of these agencies are different, each requires a rigorous safety assessment.

Early in the development of these safety assessments, it was recognized that modification of plants by the introduction of one to a few genes is not likely to substantially alter the plant's potential allergenic activity, unless the introduced gene encodes an allergen or a protein that is highly homologous to an allergenic protein. Consequently, specific guidance was developed to evaluate the possibility that any newly produced protein could be allergenic or homologous to an allergen (5). An important aspect of this holistic assessment of the allergenic potential was the recommendation to test the IgE binding properties of the introduced protein, if the protein was derived from an allergenic source or was highly homologous to a known allergen, subjects who have IgE specific to the gene source, or to a known allergen with highly similar amino acid sequence. To date, only a few human serum or clinical studies have been performed on GM crops or the introduced proteins, because most of the genes have been obtained from nonallergenic sources and the proteins have not shown significant sequences homology to known allergens. One example where human IgE binding was used involved the evaluation of soybeans modified by the introduction of the Brazil nut gene encoding a high methionine 2S albumin protein to improve the amino acid profile of soybeans for animal feed. Because the Brazil nut is a known allergenic food, the protein encoded by the gene derived from Brazil nut was assessed to determine if it encoded an allergen. This protein bound IgE from individuals with allergies to Brazil

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nuts, and skin tests confirmed it to be a major allergen (6). As a result, the potential product was never commercialized.

In another case, IgE binding studies were performed to evaluate reported reactions to the Cry9C protein in taco shells made from flour that contained StarLink corn. The Cry9C gene was obtained from a strain of *Bacillus thuringiensis*, an organism that is used as a biological insecticide in organic farming. The EPA had approved the GM product for animal feed, but had not approved it for food use, because of the apparent stability of the Cry9C protein in pepsin. When news reports indicated that some of the grain was inadvertently sold to food manufacturers and was found in commercial taco shells, a number of individuals claimed to have experienced allergic reactions to the product. Consequently, the EPA established expert panels to provide scientific recommendations regarding the potential risk of the product. The Centers for Disease Control evaluated all claims of allergy, and the FDA performed an IgE binding study to evaluate potential IgE binding to Cry9C (7). Results from those investigations were negative. However, some criticism remained, and there were questions about the equivalence of the Cry9C test material, produced in *Escherichia coli*, compared to the form of protein expressed in corn.

Scientifically meaningful IgE assays are difficult to perform, because of the low incidence of individuals with specific allergies, the low concentration of IgE in serum compared to IgG, the lack of standardized methods, and the difficulty of validating a method when no positive control sera are available to establish a value for a true positive result (7). This review will focus on appropriate methods for protein-specific in vitro IgE assays, and will present guidance regarding the development of such assays and the need for clearly defined criteria for the selection of serum donors.

Food Allergy and Diagnostic Practices

Allergic reactions to food proteins can be severe. A small number of reactions to a few of the most allergenic foods are estimated to result in 100–200 deaths in the United States each year (8, 9). Although 1–2% of adults and 4–6% of children are allergic to some food, reactions to each specific food are relatively rare, and are typically caused by the presence of one to a few highly allergenic proteins in the food (10). The mechanism of most immediate and life-threatening allergic reactions caused by the ingestion of foods is the release of potent mediators (e.g., histamine, leukotrienes, and tryptase) from tissue mast cells or blood basophils. Mediator release is triggered through allergen-specific binding of IgE on cell surface receptors (11–13). Clinical reactions vary widely between individuals and may include hives, swelling of the throat or facial tissues, vomiting, diarrhea, asthmatic wheeze, difficulty in breathing, and hypotension. In some cases, identification of the offending food is only possible through elimination diets. Accurate diagnosis often requires the expertise of a clinical allergy specialist, who will typically use controlled food challenges and/or skin prick tests (SPT) with extracts of the suspected food. Allergen-specific in vitro

serum IgE assays are often useful in confirming the causative agent, especially in young children, those with severe dermatitis, or those with severe reactions who may be at risk if orally challenged. Allergen-specific SPT and laboratory IgE tests are also frequently used to identify offending environmental allergens (pollens, house dust mites, molds). Because of the nature of the disease, food allergy is best managed through complete avoidance of the offending food.

Peanuts and tree nuts (e.g., walnut, hazelnut, almonds) have been estimated to cause allergic reactions in just over 1% of the U.S. population. A small percentage of those individuals will experience one or more severe, life-threatening reactions during their lifetime following ingestion of even small quantities of the specific food (14). Other food crops, such as rice and corn, rarely cause serious reactions, although reactions may be more common in specific restricted geographies. For example, celery and squash are rarely reported as allergens in the United States, yet in Switzerland and Germany, where raw celery root and raw squash are more commonly consumed; allergic reactions are more common and occasionally severe (15). Dietary practices are clearly important in sensitization, but there is also good evidence that environmental factors such as exposure and subsequent sensitization to cross-reactive pollen from endemic plant species may significantly increase the incidence of specific allergies (16–18).

Well-characterized and representative allergy extracts for SPT and in vitro IgE tests are essential for the accurate diagnosis of specific allergies. Although a small number of allergen extracts have been standardized internationally, standardization is typically based on the content of 1 or 2 of the major allergenic proteins, or on the overall IgE binding, or SPT reactivity of relatively uncharacterized extracts. SPT and allergen-specific IgE tests provide valuable data for the clinician; however, it is not always possible to accurately correlate quantitative results from either test with clinical symptoms. Weak to moderately positive results may be seen for individuals who are not symptomatic upon exposure to those materials. False-negative reactions are possible if the IgE epitopes recognized by an allergic individual's IgE are not present in the test material. In addition, it is not possible to quantitatively compare test results across allergens, as assay results cannot be validated to an absolute standard of clinical reactivity. Furthermore, typical in vitro IgE binding assays do not distinguish between proteins that contain only one binding site (epitope) and those that contain multiple epitopes. The former will not cause mast cell degranulation, whereas the latter will.

Because there have been significant differences in the quality and accuracy of in vitro IgE assays and allergen extracts, a scientific group consisting of representatives from clinical laboratories, allergists, allergen extract, and in vitro diagnostic testing industries have developed a guideline for assay validation and performance evaluation of allergen-specific IgE tests (19). Even though recent publications indicate relatively good agreement of results for the same sera analyzed for IgE binding to a few allergens in

assays produced by different companies, it is not possible to precisely compare results across manufacturers for most allergens (20, 21). Furthermore, even though many of the assays are calibrated to the World Health Organization standard for IgE, it is not possible to quantitatively evaluate differences in reported IgE levels between different allergens because of the complex nature of most allergens and the large variation in individual sensitivities. Allergens used in extracts for SPT and in vitro test systems are mostly from natural sources with lot-to-lot variation in the amount of specific allergenic protein, and individuals are often sensitized to more than one protein in the allergenic source. There is also considerable variation between allergic individuals regarding the specific IgE epitopes on a given allergenic protein. Ideally, each in vitro antigen-specific IgE test would be calibrated and validated so that the probability of a clinically significant reaction can be estimated. However, in practice that has only been achieved with good success for the highest levels of specific IgE to peanuts, milk, egg, fish, soybean, and wheat as measured by Pharmacia Diagnostic's (Uppsala, Sweden) CAPS system (22).

Assessment of the Potential Allergenicity of GM Crops

The primary focus for the assessment of the potential allergenicity of a GM crop is whether the introduced gene encodes an allergen or a protein sufficiently similar in sequence to an allergen that it might cause allergic cross-reactions (5, 23, 24). Recommendations developed by Metcalfe et al. (5) included a decision-tree approach with evaluation of the source of the gene, sequence similarity to known allergens, stability of the protein in pepsin at low pH and, when appropriate, serum IgE testing. If the source of the gene is allergenic, the protein is evaluated primarily by specific IgE assay using sera from individuals with clear allergies to the source material, if available. Under specific circumstances, SPT and food challenges were also recommended (5); however, practical, legal, and ethical considerations severely limit the potential use of such tests (25). Additionally, the amino acid sequence of all proteins, regardless of the source of the gene, is compared with those of known allergens. If the sequence is similar to an allergen, serum from individuals allergic to the source of the allergenic protein should be used to evaluate IgE binding. Recent guidelines by the Codex Alimentarius Commission, the body responsible for international harmonization of the safety assessment of foods, has adopted a holistic, weight-of-evidence approach rather than a decision-tree approach, and affirms that all test methods and decision criteria be based on sound science (26). The remainder of this paper will focus on test material characterization, criteria for serum donor selection, and methods and evaluation of specific IgE immunoassays.

Test Material Characterization

Test proteins used to evaluate IgE binding must be characterized to demonstrate that they are equivalent to the protein produced in the GM crop. In some cases, the concentration of the introduced protein is present at only a few parts per million in the crop or in the food produced from the crop (1), and cannot be practically extracted in sufficient quantities to perform all tests. In such cases, a heterologous expression system (e.g., bacteria, yeast, tobacco plants, or plant plastids) may be used to provide higher quantities, concentrations, and purity of the protein. When using heterologously expressed proteins, the identity, sequence, mass, and glycosylation status must be equivalent to the protein produced in the GM plant. The relevant biological or enzymatic activity of the purified protein should be tested to evaluate native conformation. In addition, the concentration and purity of the material should be tested. Typically, proteins purified from plant sources, or heterologous expression systems, are 60–95% pure. The contaminants may include allergenic proteins from the source, which will need to be evaluated in developing IgE binding assays specific to the introduced protein.

In addition to pure protein, extracts of the GM plant material and conventionally bred plants may be used as the IgE binding target either to evaluate potential changes in endogenous allergenicity (27, 28) or to confirm IgE binding results to the purified protein. In such cases, protein-specific animal sera, produced by immunizing laboratory animals with purified protein, should be used to confirm the presence of the introduced protein in the appropriate extract and may also be used for quantification of the protein. Such antibodies will also be useful for testing the presence of the protein in the IgE assays described below and in demonstrating the immunoreactivity of the protein.

Serum Donors for IgE Testing

As envisioned by the original allergenicity assessment scheme, a major determinant in successful serum IgE testing is the selection of appropriate donors to provide test and control sera (5). Serum (or plasma) samples from individuals who are clearly allergic to either the donor organism or to the organism that produces the allergen that is similar in sequence to the introduced protein will be used as test sera. If the prevalence of allergies to that source is very low, 5 test sera may be sufficient for an adequate safety test, providing ca 67% probability of detection if the frequency of sensitization to the particular protein is >20%, or 95% probability if the frequency is >50% (5). If the source commonly causes allergies, or the matched protein is a major allergen, the use of serum from 14 donors with allergies to the source material would provide a 95% probability of detection if the prevalence of sensitization in the population were 20%, based on binomial probability theory (5).

Experienced clinical allergists should evaluate the allergic history of potential serum donors, perform any in vivo tests

(SPTs or food challenges) if appropriate, and evaluate *in vitro* allergen-specific IgE test results. Permission to perform tests and obtain sera used in the study should be obtained under informed consent and institutional review board rules of the local government and medical facilities. Allergic symptoms upon allergen exposure should be immediate (<2 h), specific, and reproducible. For food allergies, individuals with systemic reactions, or symptoms with involvement of 2 or more organs (e.g., hives or facial swelling and difficulty breathing, vomiting, diarrhea, rhinitis, or loss of blood pressure) are preferred as donors. Their serum (or plasma) samples should be tested and confirmed positive for IgE binding to proteins of the allergen as measured by commercial assay (e.g., class III or higher, >3.5 kU_A/L, using allergen-specific Pharmacia UniCAP tests). If food challenges or SPT results are not available or are impractical, but the subject has a convincing clinical history and allergen-specific *in vitro* assay results of very high levels (class IV, V, or VI by Pharmacia UniCAP), this sera may be useful if sufficient numbers of sera from challenge test-positive donors are not available. Clinical symptoms for airway allergens may be less clear, but would typically include rhinitis, conjunctivitis, and/or wheezing within minutes after exposure. Direct challenge testing is rarely performed for inhalation allergy. SPTs and allergen-specific *in vitro* IgE measurements are very important in the diagnosis, though the levels of IgE by commercial test assay should be in the high or very high range (e.g., greater than class III by Pharmacia UniCAP). Symptoms for contact allergens are highly varied, and patch testing may not be specific because of hyper-responsiveness of the skin. Therefore, specific *in vitro* IgE test results are quite important for diagnosing some patients.

Additional sera from individuals with demonstrated allergies to other allergens, who do not have clinical allergies to the allergen of interest or specific IgE to the known allergenic protein, should be used as negative controls for the allergen of interest. Sera from individuals without any significant allergies may also be used as negative controls. If the IgE assay results of the negative controls indicate binding to the test (GM) protein, the specificity of the assay should be questioned. It must be recognized that the volume of serum or plasma available from each donor will typically be limited to no more than a few milliliters, which limits the type and number of tests that may be used in these assessments.

In vitro Protein-Specific IgE Assays

Since the early 1990s, IgE-specific Western blots and enzyme-linked immunosorbent assays (ELISAs) have frequently been used to identify allergenic proteins in foods and environmental agents. Each has specific merits and limitations. However, these methods may be useful for assessing potential IgE binding to the proteins introduced into GM crops. It should be recognized that assay results would generally be qualitative or semi-quantitative, as it is impractical to validate the materials and methods to the degree needed for absolute quantification (19). Additionally, the

sensitivity and specificity of these immunoassays must be evaluated during assay development to ensure that the results are meaningful regarding the potential allergenicity of the novel protein.

Western Blotting

Direct Western blotting may be used to confirm that serum IgE binds to the protein of interest rather than to other constituents in the complex mixture of proteins present in extracts of plant material used in SPT or ELISA tests or in whole foods. Proteins are separated by electrophoresis through a gel matrix, most commonly polymerized acrylamide (PAGE), with sodium dodecyl sulfate (SDS) detergent and a reducing agent (dithiothreitol or beta-mercaptoethanol) to denature the proteins and provide a uniform negative charge to facilitate separation. The proteins are then transferred to a nitrocellulose or polyvinylidene difluoride (PVDF) membrane and incubated with a blocking agent [bovine serum albumin (BSA), gelatin or nonfat dry milk (NFDM)] to prevent nonspecific antibody binding. Blocked membranes are incubated with test or control sera to differentiate specific IgE binding from nonspecific binding. After washing, bound IgE molecules are detected with a specific antihuman IgE secondary antibody that is labeled with a radioactive isotope and detected by exposing X-ray films (29), or a reporter enzyme [alkaline phosphatase or horseradish peroxidase (HRP)] and substrate for colored or luminescent detection (30). The sensitivity of detection may be significantly increased by the use of amplification steps (biotinylated anti-IgE followed by NeutrAvidin-HRP, or anti-IgE followed by biotinylated protein G, and then NeutrAvidin-HRP). Although antigen-specific Western blot assays that use serum IgG from immunized animals for detection of proteins may detect picogram levels of antigen, the relatively low abundance of serum IgE even from highly allergic subjects limits detection to low nanogram levels of antigen, even with amplified detection using chemiluminescence. Lower sensitivity is partly due to nonspecific background binding of detection reagents. This background may be reduced by the choice of blocker and by preincubating blocker and secondary antibodies and NeutrAvidin in blocker before adding to the membrane. The specific goat or rabbit anti-IgE detection IgGs may also contain IgG specific to other proteins in plant extract samples.

An example of differential blocking efficiency is shown in Figure 1. Phosphate-buffered saline (PBS) extracts of defatted peanut flour, full-fat soybean flour, full-fat corn flour, and purified recombinant house dust mite allergen Der f 2 were prepared in Laemmli buffer with 2-mercaptoethanol and heated at 100°C prior to loading on 10–20% Tris glycine gels for electrophoresis. Proteins were electrophoretically transferred to Immobilon-P membranes after electrophoresis. Nonspecific antibody binding was blocked with either 2% NFDM in PBS plus 0.05% Tween 20 (PBST), with 2% BSA in PBST or with 0.2% Tween 20 in PBS.

Plasma from a peanut-allergic donor (No. 297) with no known allergic reactions to soybean or corn, and a Pharmacia

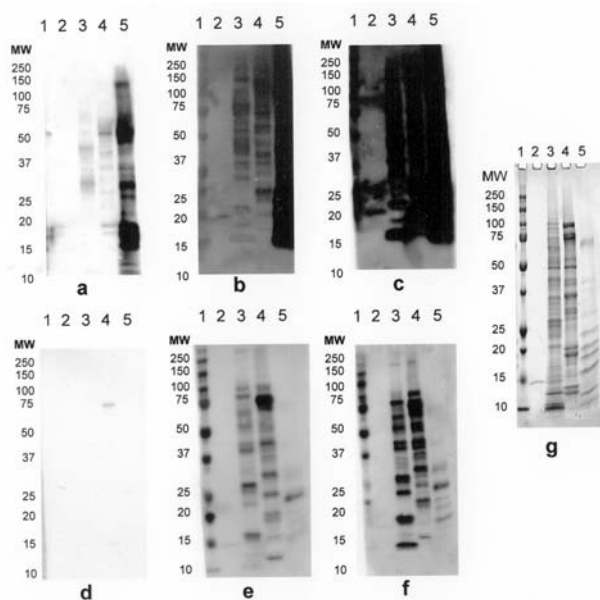


Figure 1. Blocking conditions for direct antigen-specific IgE binding in Western blots. Lanes 1–5: molecular weight markers, Der f 2 from Indoor Biotechnologies (Charlottesville, VA; 400 ng), maize extract (5 μ g), soybean extract (5 μ g), and peanut extract (1.25 μ g), respectively (total protein). Samples were separated by electrophoresis in 10–20% Trisglycine SDS-PAGE (Invitrogen, Carlsbad, CA; No. EC61355) after reducing (5 min at 100°C) in Laemmli buffer with 2-mercaptoethanol (2.5%) prior to electrotransfer to PVDF membranes. Blots a and d were blocked with 2% NFDm (local brand); blots b and e with 2% BSA (Sigma, St. Louis, MO; No. A-3675); and blots c and f with 0.2% Tween 20 (BioRad, Hercules, CA; No. 170-6531). A colloidal blue stained gel (Sigma; No. B2025) is shown in g. Blots a–c were incubated with peanut-allergic plasma No. 297 diluted 1:10 in appropriate blocker ca 60 min before adding to membranes. Blots d–f were incubated with aeroallergen-specific plasma No. 300 diluted 1:10 in appropriate blocking solution ca 60 min prior to adding to membranes. Membranes were incubated in plasma for ca 2 h. Unbound IgE was removed by 4 washes in PBST. Bound IgE was detected by using biotinylated goat anti-IgE (KPL, Gaithersburg, MD; No. 16-10-04) diluted 1:4000 (v/v) in appropriate blocker for 1 h, followed by 4 washes in PBST, then incubation for 1 h in 1:10 000 diluted NeutrAvidin-HRP (Pierce Chemicals, Rockford, IL; No. 31001) that was diluted in the appropriate blocking solution. After 4 washes in PBST, and membranes were wet with ECL reagent (Amersham BioSciences, Piscataway, NJ; No. RPN2106), covered in plastic and used to expose Hyperfilm (Amersham BioSciences; No. RPN3114K) a few seconds to 2 h. Films developed after 15 s exposure are shown.

UniCAP class V score for peanut and class II for soybean, was used as a positive control for peanut extracts. Plasma from an aeroallergen-sensitized donor (No. 300) with no known allergic reactions to peanut, soy, or corn, and a Pharmacia

UniCAP class 0 score to peanut and soybean, was used as a negative control. Both plasma were diluted 1:10 (v/v) in the corresponding blocking solution and mixed for 30 min at room temperature to let any IgE specific to the blocking protein bind in solution. The diluted plasma was added to the membrane and incubated with mixing at room temperature for 2 h. Membranes were washed 4 times with PBST, and then incubated for 1 h with biotinylated goat antihuman IgE diluted 1:4000 in blocking solution. After 4 washes in PBST, membranes were incubated for 1 h with NeutrAvidin-HRP that was diluted 1:10 000 (v/v) in blocking solution. After 4 washes in PBST, membranes were wet with freshly mixed electrochemiluminescence (ECL) reagent at room temperature (RT), placed between clear plastic transparency sheets, and visualized by exposing Hyperfilm for various times. A 15 s exposure is shown in Figure 1. The background of the membranes blocked with NFDm (Figure 1, a and d) was clear and bands corresponding to the expected major allergen (Ara h 2) of peanut (31) were obvious (lane 5 of Figure 1a) for the peanut-allergic plasma. These bands were lacking from the membrane incubated with the non-peanut allergic plasma (Figure 1d). Although there were faint additional bands in peanut, soybean, and corn extracts (Figure 1a), the level of detection of bands in the irrelevant allergens soybean and corn were very low compared to 5 or 6 bands in peanut, the specific allergen for this individual. In contrast, many high-intensity irrelevant bands, including marker proteins, were detected in blots blocked with 2% BSA (Figure 1, b and e) and with Tween 20 at 0.2% (Figure 1, c and f). A gel loaded identically to those blotted was stained with colloidal blue (Figure 1g) to demonstrate the protein loading. These results demonstrate that an appropriate blocking reagent is necessary when highly sensitive detection methods are used in order to avoid false-positive results. This nonspecific binding may be due to protein-protein interactions, or very low affinity antibody binding.

The sensitivity of the Western blot may be defined as the minimum quantity of a particular allergen that is clearly demonstrated to bind IgE from individuals with proven clinical allergies, under conditions that do not allow binding by IgE from those who are not clinically reactive to the protein. Based on limited testing with 2% NFDm as described above and IgE in plasma-diluted 1:10 from 2 individuals with clinical histories of peanut allergy, the sensitivity is <40 ng Ara h 2 per sample lane. Under the conditions used, there was no apparent binding of IgE from 3 individuals with no known reactions to peanuts (data not shown).

In some cases, direct binding Western assay results may appear to be specific based on intensity, but the results may not correlate with the clinical history of the donor, as shown for donor No. 300 with soy proteins (Figure 1, e or f). In such cases, the specificity of IgE binding should be tested with specific protein inhibitors (32). An example of an inhibition experiment is shown in Figure 2. Proteins in soybean extract, peanut extract, and purified Ara h 2 isolated from peanuts were separated by electrophoresis and transferred to PVDF membranes as described for the direct Western. All

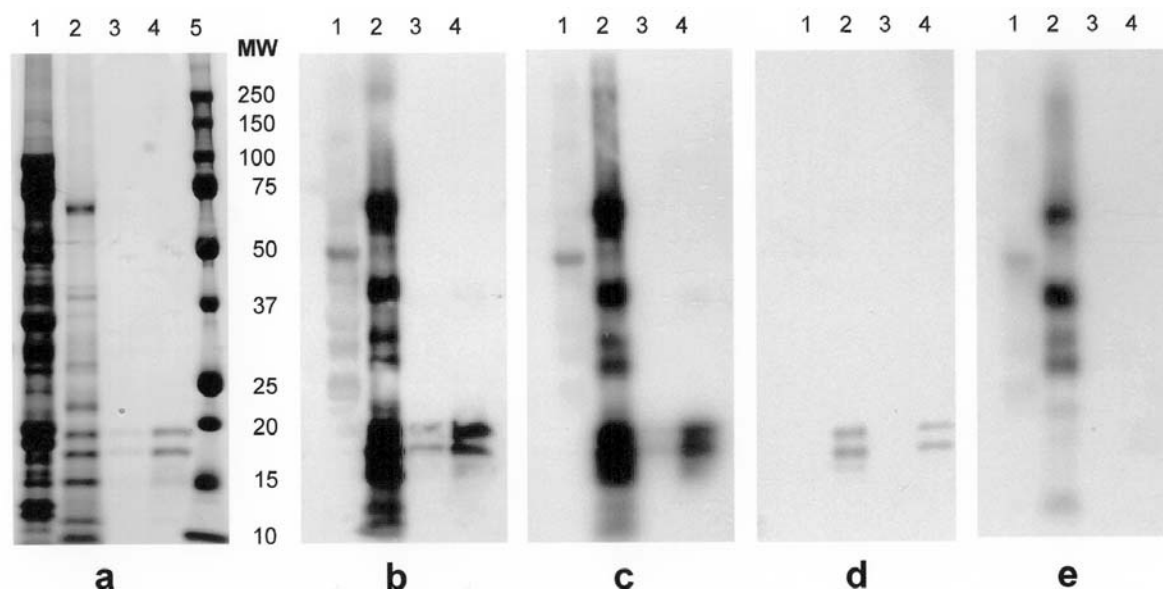


Figure 2. Inhibition of IgE binding to specific proteins in Western blots. Soybean extract (10 µg), peanut extract (2.5 µg) allergens, Ara h 2 (40 ng) or Ara h 2 (400 ng), and molecular weight markers (lanes 1–5, respectively) were separated by electrophoresis and blotted onto PVDF membranes as in Figure 1. Proteins in one gel were stained with colloidal blue (a). All membranes were blocked with 2% NFDm. Western blots were performed with peanut-allergic plasma sample No. 297 in 2% NFDm, but without inhibitor (b) or with 1 of 3 inhibitors: 160 µg Rubisco (c), 160 µg peanut extract (d), or 160 µg Ara h 2 (e) diluted in 2% NFDm. Inhibition was accomplished in solution at RT for 2 h before addition of plasma to membrane. After removing unbound IgE by washing in PBST, bound IgE was detected as in Figure 1.

membranes were blocked with 2% NFDm in PBST. Then, samples of plasma from peanut-allergic donor No. 297 were pre-incubated with blocker or with 40 µg/mL of 1 of 3 inhibitors (Rubisco, peanut extract, or Ara h 2) in PBST with 2% NFDm for 45 min at RT. Preblocked membranes were incubated for 2 h in the plasma with or without inhibitor. After washing, detection was accomplished as outlined for the direct binding experiment. One gel was stained with colloidal blue (Figure 2a) to visualize the protein bands. Direct binding with plasma pre-incubated in blocker alone demonstrates clear IgE binding to purified Ara h 2 and Ara h 2 in peanut extract, along with a few additional peanut proteins, and some lighter bands in soy extract (Figure 2b). Pre-incubating the plasma with Rubisco did not significantly alter that binding pattern (Figure 2c). However, pre-incubating the plasma with peanut extract markedly reduced IgE binding to Ara h 2 and blocked IgE binding to other peanut and soybean proteins (Figure 2d). Inhibition of binding to the strong soybean band suggests that protein is cross-reactive to a peanut allergen. The residual Ara h 2 bands demonstrate incomplete inhibition, because of insufficient Ara h 2 in the inhibitor. Pre-incubation of plasma with purified Ara h 2 blocked binding to Ara h 2 in peanut and the purified protein lanes, but did not block IgE binding to other peanut proteins or the most prominent band in soybean extract (Figure 2e). In many cases, inhibition tests similar to those described here are necessary to demonstrate the specificity of IgE binding.

A limitation of Western blotting is the uncertainty of whether conformational IgE epitopes are represented by the

protein on the blot, especially from electrophoresis in denaturing conditions. Depending on the structure of the protein, it may refold to some degree once wet with blocker, to an extent that will allow some IgE binding to conformational epitopes. However, many conformational epitopes will not be represented in the Western blot as they require secondary, tertiary, or quaternary structures that require appropriate disulfide bond formation or the presence of additional peptide subunits. An important advantage of Western blotting is the identification of the apparent molecular weight of the protein, often confirming the specific allergen, whereas other immunoassays such as ELISA that typically use native protein as a target, do not allow the identification of the allergenic protein unless a highly purified protein is used as the target. Western blots of proteins separated by nonreducing SDS-PAGE, or native gels (without SDS or reducing agent) may also prove useful to evaluate IgE binding to conformational epitopes (33). There are many variations on methods of separation, transfer of proteins to the membrane, nonspecific blocking, dilution of sera, selection of appropriate positive and negative sera, and detection methods that will affect the utility of these assays (30).

ELISA

Quantitative or semiquantitative measurement of allergen-specific IgE is frequently made using this simple immunoassay technique common to most biochemistry laboratories. Proteins may be bound to polystyrene microtiter plates in high pH carbonate buffer in native conformation, to

Table 1. Relative quantities of peanut extract-specific IgE measured by direct ELISA^a

Plasma donor	Clinical allergy	Plasma dilution, OD 450 nm		
	Peanut (Y/N)	1:40	1:160	1:320
No plasma ^b			(0.188)	
297	Y	2.386	1.177	0.69
299	Y	2.208	1.066	0.67
300	N	0.086	0.040	-0.003
708	N	0.051	-0.009	-0.006
709	N	0.024	-0.008	-0.01
Avg. negative control		.054	.019	-0.006
SD		.031	.018	0.004
Positive detection level		0.147	0.073	0.006

^a Plasma samples were tested in triplicate at 3 concentrations to measure IgE binding to peanut proteins coated on Nalge microtiter plates.

^b The "No plasma" average OD reading, background level of detection caused by biotinylated goat anti-IgE plus NeutrAvidin coupled to HRP, was subtracted from each raw OD value before calculating the average, SD, and positive detection level, which was calculated as the average negative control plus 3 SDs (Nos. 300, 708, and 709).

act as the IgE binding target (34). It is expected that many, if not most, conformational epitopes are available as targets under these conditions, though internal or linear epitopes may be masked. Complex mixtures of proteins in crude extracts of the plant material, proteins purified from such extracts or from heterologous expression organisms such as *E. coli*, may be used as the target antigen.

Assay development requires the use of positive and negative controls to establish the optimum protein coating concentrations, serum dilutions, and limits of detection or quantification. Development of valid assays requires sera from individuals with clear clinical allergic responses to the test material and sera from individuals without clinical responses to the test material. As with the Western blot assays, the negative control donor population should include those who are nonatopic (without known allergies and without elevated total IgE) as well as individuals having strong allergic reactions to other unrelated organisms, or individuals with elevated total IgE. If the assay is to test IgE binding to a specific protein produced by an organism, it is often useful to first develop an ELISA against an extract of the organism and to perform Western blot assays to evaluate the specificity and antibody titer of the sera before attempting to develop an ELISA to the purified protein. An example of the development process follows.

To aid in the development of specific, direct IgE ELISAs for the peanut allergens Ara h 1 and Ara h 2, an assay for peanut extract was used to optimize detection and identify appropriate control sera. Plasma samples were collected by SeraCare Life Sciences (Oceanside, CA), an FDA-licensed biological supplier, from human subjects having specific clinical histories of allergic reactions. Three donors (Nos. 300, 708, and 709) reported allergic reactions to various pollen or environmental allergens, but not to soybean or peanut allergens. These samples tested negative (class 0) by Pharmacia Diagnostics in vitro IgE assays for both soybean

(f14) and peanut (f13) allergens. Two subjects (Nos. 297 and 299) had histories of serious allergic reactions following the ingestion of peanuts, but not soybeans. Test results from Pharmacia CAPS tests for soybean were equivocal for Nos. 297 and 299 (class II and III, respectively). Both samples were strongly positive (class V) for peanut allergen.

Preliminary tests were used to determine the optimum concentration of protein (5.0 µg/mL) for coating of microtiter plates (34). Peanut extracts were diluted in pH 9.6 carbonate buffer, and incubated in plates overnight at 4°C in a humidity chamber to coat the wells. The coating solution was removed by washing with PBST, and nonspecific binding was blocked with 2% NFDM in PBST. Plasma samples were diluted 1:40, 1:160, and 1:320 (v/v) in RT blocking solution 30 min before addition to aspirated sample wells. Plates were incubated for 2 h in a humidity chamber at RT. Additional wells coated with peanut extract were incubated with blocker alone to serve as a control for secondary antibody background binding. Wells were washed 3 times with PBST before the addition of biotinylated goat anti-IgE (KPL, Gaithersburg, MD; No. 16-10-04), which was diluted 1:4000 (v/v) in PBST. Plates were incubated for 90 min at RT, and then washed 3 times with PBST. NeutrAvidin-HRP (Pierce Biotechnology, Inc., Rockford, IL; No. 31001) was diluted 1:8000 (v/v) in PBST, added to sample wells, and incubated for 60 min at RT. Plates were washed 3 times with PBST before addition of 100 µL substrate [tetra-methylbenzidine (TMB) from KPL; No. 50-76-00] at RT. Reactions were stopped after 10 min of incubation at RT by addition of 50 µL 6N sulfuric acid and the optical densities (ODs) were read at 450 nm in a PowerWaveX plate reader (Bio-Tek Instruments, Inc., Winooski, VT). The values in Table 1 reflect the average of 3 independent wells, with the average background (no-plasma wells) subtracted from the raw OD readings. Results for plasma from non-peanut-allergic individuals (Nos. 300, 708, and 709) indicate low levels of IgE binding at the highest

Table 2. Relative quantities of specific IgE binding to Ara h 1, Ara h 2, and Rubisco as measured by direct ELISA^a

Clinical allergy		Purified protein coating test wells (plasma dilution 1:40; OD 450 nm)		
Plasma donor	Peanut (Y/N)	Ara h 1	Ara h 2	Rubisco
No plasma ^b		(0.061)	(0.062)	(0.090)
297	Y	1.264	1.184	0.078
299	Y	1.453	1.746	0.081
300	N	0.069	0.090	0.090
708	N	0.046	0.074	0.069
709	N	0.046	0.067	0.043
Avg. negative control		0.054	0.077	0.072
SD		0.014	0.012	0.018
Positive detection level		0.096	0.113	0.126

^a Plasma samples were tested in triplicate at 3 concentrations to measure IgE binding to each of 3 proteins.

concentration of plasma, tested when compared to the no-plasma sample wells. At higher dilutions of plasma the values drop to the level in wells that did not receive any plasma. However, peanut-allergic plasma (Nos. 297 and 299) exhibited high OD readings even when diluted to 1:320 (Table 1), showing high sensitivity.

An essential question in assay development is where to set the minimum level for positive detection. Under ideal circumstances, a validation would be performed with sufficient numbers of sera from individuals with and without specific allergic reactions to determine optimum cutoffs by statistical evaluation of the positive and negative predictive values. However, that is not expected to be the case when GM proteins are assessed, when relatively low numbers of individual serum donors with relevant allergies are likely to be available. In such cases, cutoff values may be based on the average value from nonallergic control sera, plus either 2 or 3 standard deviations (SDs) of the values used to calculate the average (19, 35, 36). In the example shown in Table 1, the average for the 3 negative plasma samples at the 1:40 dilution is 0.054 OD units, the SD is 0.031 OD units, and the cutoff (average plus 3 SDs) would be 0.147 OD units. Results > 0.147 OD units for the test sera may be considered positive.

Assays using pure proteins can behave differently from those using whole extracts. In the assessment of the potential allergenicity of GM proteins, immunoassays with pure proteins will be used to measure IgE specific to the novel protein. In experiments reported here, the same 5 plasma samples were used to develop direct-binding ELISA assays for the peanut allergens Ara h 1 and Ara h 2 as well as a negative control protein, Rubisco, which was isolated from spinach leaves. Optimum coating concentrations were determined for the purified peanut-allergens (2 µg protein/mL coating buffer) based on IgE binding from the peanut-allergic

donors. Rubisco was prepared at the same concentration for coating the microtiter plates, as positive sera are not available and there are no reports that Rubisco is an allergen. Because the concentration of IgE specific to the individual proteins is expected to be less than for the whole extract of peanut, lower dilutions of plasma were used (1:10, 1:40, and 1:200). Otherwise, the procedure was identical to that used to assess binding to peanut extract.

OD values obtained from 1:40 dilution of plasma are shown as representative of IgE binding to each of the 3 purified proteins (Table 2). The results of non-allergic plasma samples (Nos. 300, 708, and 709) were used to calculate the cutoff limit for positive detection (average plus 3 SDs) for each assay, 0.096 OD units for Ara h 1 ($n = 3$), and 0.113 OD units for Ara h 2 ($n = 3$). The cutoff limit for Rubisco ($n = 5$) was calculated to be 0.126 OD units. Results for peanut-allergic plasma samples Nos. 297 and 299 (OD > 0.23, data not shown), when the plasma samples were diluted 1:200, were markedly above the positive detect level for these peanut allergens. Results from the 5 plasma samples in the Rubisco assay were all < 0.1 OD units, indicating little or no IgE binding to the protein. Such results would be expected in cases where a protein is not allergenic, or when tests were performed by using samples from individuals without allergic responses to the test material.

Investigators working on the evaluation of claims of allergic reactions to a GM protein, Cry9C, in taco shells did not have any positive IgE serum samples to establish their assay, a situation similar to that for Rubisco as described above (7). There were no identified individuals who were allergic to the source organism, *B. thuringiensis*, or to the protein encoded by the inserted gene before the study. Therefore, investigators used serum samples collected before commercial production of StarLink as well as freshly

collected sera from non-atopic individuals as 2 populations of negative controls. In that case, goat anti-Cry9C was used to validate protein loading and detection from the microtiter plates under the assay designed to measure human anti-Cry9C IgE. None of the results with sera from individuals who claimed to have reacted to the ingestion of corn products made with Cry9C were higher than the negative controls. Based on the apparent lack of specific IgE, the appropriate conclusion is that none of the individuals were sensitized to the Cry9C protein. However, some individuals have continued to question the results of the study. One claimed to have experienced repeated reactions to food containing StarLink corn, but when the appropriate blinded food challenge was performed, that individual did not react to high-level oral doses containing StarLink (37).

Questions regarding the specificity of direct-binding ELISA assays may be addressed through inhibition experiments similar to those described for Western blots. Sera would be incubated with various concentrations of the protein used to coat the plates before addition to sample wells. Additionally, a negative control protein should be tested as an inhibitor to verify the specificity of inhibition. If the specific inhibitor does not reduce binding to the solid-phased protein in the microtiter plate, binding should be considered to be nonspecific.

If the assays are being performed to evaluate potential cross-reactive binding of IgE to the GM protein compared to an allergen that is similar in sequence, additional wells should be coated with the known allergen. Then direct binding to both the GM protein and the allergen may be compared. In such cases, reciprocal inhibition tests should be used to evaluate the similarity or differences in binding efficiency to the introduced GM protein and the proven allergen.

Conclusions

The safety assessment of GM crops includes evaluation of the potential allergenicity of the novel introduced proteins. As articulated in 1996, there are specific circumstances when sera (or plasma) from subjects with specific allergies should be used to determine whether the protein is likely to bind IgE and possibly could result in allergic reactions in those with pre-existing allergies (5). There are clear instances when serum testing is reasonable and is possibly the only way to complete the assessment of the potential allergenicity of proteins introduced into GM crops. The 2-S albumin protein from Brazil nuts was first identified as an allergen when such tests were performed to assess potential IgE binding to the protein that had been introduced into soybeans (6). In that case, the positive *in vitro* binding of IgE with sera from Brazil nut-allergic individuals was confirmed by SPT with Brazil nut extract and the GM material. The claims of allergic responses to Cry9C in StarLink corn were investigated by serum testing. In that case, negative results indicated that the protein was not

the likely cause of the claimed allergic responses (7). There are occasional published reports of allergens or suspected allergens being introduced into GM crops (38–40). In those cases serum from individuals with appropriate allergies would be useful in evaluating the potential risk associated with having those proteins introduced into commodity crops. The methods and potential technical issues discussed here deserve additional consideration in cases where protein-specific IgE binding assays are needed to evaluate the potential that a transferred protein is cross-reactive (41, 32).

Both direct and inhibition Western blot assays and ELISAs may be useful in evaluating the potential allergenic activity of the transferred protein. These assays must be designed to be highly specific and sensitive. Demonstration of assay validity also requires characterization of the test materials and demonstration of immunoactivity in the assay format. The use of Western blotting to evaluate IgE binding to the introduced proteins in GM crops requires preliminary development to optimize blocking reagents, detection methods, and loading of test materials to optimize conditions of direct binding or inhibition Western blot assays to establish sensitivity and specificity. As demonstrated in the examples shown, it is clear that failure to optimize Western blots methods could lead to false-positive IgE binding. Further, it is important to include positive allergen controls for the individual serum donors to verify their allergic status and provide an example of positive allergen detection. Although Western blot assays can confirm the identification of the specific protein in a complex mixture that is bound by IgE, a quantitative measurement of binding is required to help evaluate the relevance of any identified binding. However, little work has been done to validate any quantitative immunoassays for demonstrating the lack of allergenicity. Under ideal circumstances, a full validation of the immunoassays would be undertaken prior to the use of the assay (19). However, that requires the identification of individuals with proven allergies to the specific allergen of interest, something not expected for proteins introduced into GM crops. Further, it requires a large population of allergic and nonallergic individuals to establish a biologically meaningful cutoff. Under such circumstances, it seems reasonable to use a specific ELISA with pure preparations of the introduced proteins as the target and a criteria of the average negative control value plus 3 SDs from the negative control population as a conservative estimate of a positive result. In circumstances where the specificity of binding is not clear, an inhibition ELISA should be used to confirm the specificity of binding.

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