

Development and Use of an ELISA Test to Detect IgE Antibody to Cry9c following Possible Exposure to Bioengineered Corn

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Key Words

IgE · Cry9C · Allergy · Corn

Abstract

Background: Starlink™, a variety of corn genetically engineered to contain the insecticidal protein Cry9c, had not been approved for human consumption because it possessed some characteristics associated with allergenic proteins. However, in the fall of 2000 *cry9c* DNA was detected in several corn-containing products, suggesting that Starlink corn had entered the human food supply. Subsequently, consumers, following consumption of corn products, reported a number of adverse health events, possibly consistent with allergic reaction.

Methods: To investigate the possibility of allergic reactions due to Cry9c in these consumers an ELISA test was developed for the purpose of detecting IgE antibodies to Cry9c and blood samples were taken from a total of 18 people who self-reported allergic reactions. Sera collected prior to the 1996 development of Starlink were used as negative controls. **Results:** None of the adverse event sera were found to be reactive with recombinant Cry9c antigen, based on comparison with normal controls. Although a known human positive control serum

containing IgE specific for Cry9c was not available, other controls were incorporated into the ELISA protocol, including the use of sera from subjects allergic to other allergens and their homologous antigens (cat, grass, peanut) to validate the IgE detection reagents. **Conclusions:** While the results do not support the likely occurrence of allergic reactions to Cry9c, such reactions cannot be ruled out, nor can the possibility that sera might react with unique glycosylated epitopes of Cry9c that may be expressed in the corn plant/seed.

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Introduction

This article describes the procedures used to investigate reported adverse health effects linked to consumption of foods allegedly containing an ingredient produced by recombinant DNA technology, namely, Starlink™ corn. Details regarding the circumstances surrounding the presence of Starlink in the human food supply have been previously reported [1]. Starlink is one of a number of bioengineered plant products containing insecticidal proteins derived from *Bacillus thuringiensis*. In this case, the protein Cry9c has been genetically inserted in order to

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convey resistance to several agricultural pests. Starlink was not licensed for human consumption, because it exhibited some physical/chemical properties that have been associated with known food allergens [2–4]. In September 2000, *cry9c* DNA was detected first in taco shells and then in other corn products. This resulted in several widely publicized food recalls. These reports lent credibility to the possibility of extensive co-mingling of Starlink with other varieties of corn. Subsequently, the Food and Drug Administration (FDA) began receiving reports of adverse health effects from consumers who had eaten food products containing corn.

In order to investigate the relationship between the presence of Cry9c in the food supply and the reports of human illness after consuming corn products, we reviewed all adverse health events reported to the FDA between July 1, 2000, and November 30, 2000, involving the consumption of a corn product. We developed a working case definition that included signs and symptoms consistent with systemic anaphylaxis (e.g., dizziness, weakness, or loss of consciousness). Dermatological or oropharyngeal (hives, rash, pruritis, oropharyngeal tingling or swelling) and gastrointestinal symptoms (vomiting, diarrhea, abdominal cramping) were also considered provided they occurred within 12 h of product consumption, involved only 1 individual among meal companions, and could not be explained by a pre-existing medical condition. Of 51 adverse events reported, 23 did not meet these criteria. Of the 28 individuals meeting the case definition, 24 completed questionnaires. Ten of the 24 experienced symptoms within 1 h of eating and most reported multiple symptoms. Nineteen of the 24 were either self-treated or physician treated for symptoms of allergic reaction. Two people were hospitalized. Seventeen of the 24 provided serum samples for possible antibody determination. Subsequently, one additional report was discovered and serum collected, resulting in a final total of 18 sera.

Based on the initial findings, development of a laboratory method to detect serum antibodies was undertaken to further assess the relation between allergic manifestations and the Cry9c protein. The goal of this research was to determine if we could detect a difference in immune responsiveness to the Cry9c protein among differentially exposed groups using an ELISA method. Results of the serum test would indicate if individuals had specific IgE antibodies to Cry9c, suggestive of hypersensitivity.

Materials and Methods

Adverse Event Sera

Venous blood was collected from individuals reporting adverse reactions deemed to be clinically consistent with an IgE-mediated reaction, after receiving informed consent of the individuals under a protocol approved by the CDC Institutional Review Board. Blood was collected in 5 ml Vacutainer® serum collection tubes. Blood was allowed to coagulate and serum obtained by centrifugation at 1,500 g. Serum was divided into 0.5-ml aliquots and kept at –20°C until the time of testing. Blood was drawn between 2 days and 2 months following the reporting of an adverse event. Serum samples were collected and blind-coded by CDC personnel.

Normal Control Sera

Negative control sera were defined as sera obtained prior to the marketing or production of StarLink corn. These sera were obtained from banked specimens from CDC Epidemic Intelligence Service officers (EIS) officers collected prior to 1996. Serum samples from EIS officers were interspersed among the adverse event sera for purposes of coding.

Additional Control Sera

Other control sera were also used. These included sera from individuals with high (3,666–11,590 IU/ml) total IgE levels (atopic sera) and sera from individuals with clinically diagnosed allergies (medical history and/or skin test) to cat and June Grass containing specific IgE levels of >100 kIU/l. These sera were provided by IBT Reference Laboratories (Lenexa, Kans.) and lacked personal identifiers. Sera from peanut-allergic patients were obtained from the University of Nebraska Food Allergy Research Program (Lincoln, Nebr.). Specific IgE levels available for peanut sera ranged between >100 kIU/l and 3.13 kIU/l. Clinical allergies were defined based on medical history and, in some cases, skin tests. A purified IgG fraction of a goat anti-Cry9c antibody, produced against recombinant Cry9c, was provided by Aventis Crop Sciences (Research Triangle, N.C., USA).

Antigens

Recombinant Cry9c (r-Cry9c), produced in *Escherichia coli*, was provided by Aventis Crop Sciences. The recombinant protein was the insecticidal fragment of Cry9c containing amino acids 44–666, the same sequence as the protein produced in Starlink corn. The protein concentration was determined spectrophotometrically and purity was assessed by SDS-PAGE analysis on a 4–20% gradient gel. Partial sequence analysis of SDS-PAGE bands was performed by mass spectrometry [5]. Reactivity of the r-Cry9c bands with the hyperimmune goat IgG (diluted 1:1,000) was assessed by Western blot. The reaction of this serum with corn-expressed Cry9c was assessed by blotting against an extract of corn flour made from 100% Starlink™ corn or corn flour made from corn produced prior to the commercial release of Starlink. Extracts were produced by extracting 1 g of flour with 10 ml phosphate-buffered saline (PBS). On gels for Western blots, 10 and 20 µg of protein were loaded on adjacent lanes of a 4–20% gradient gel. For blotting, proteins were transferred to membranes using a tank method. As a control, duplicate blots were incubated with normal goat serum, diluted 1:1,000. Both sets of blots were then developed with peroxidase-conjugated donkey anti-goat IgG (Jackson ImmunoResearch, West Grove, Pa., USA) and 4-chloro-1-naphthol (Sigma, St. Louis, Mo., USA). The Cry9c protein level in corn flour was determined by ELISA to be 19.7 µg/g [6]. Cat and rye

grass allergens were provided by the FDA, Center for Biologic Evaluation and Research. These antigens are extracts used as reference allergens for human skin prick testing. Peanut extracts were made by suspending defatted peanut flour in 1 M NaCl-20 mM sodium phosphate (pH 7.0) (1:20 wt/vol ratio), and shaking for 1 h at 37°C. The crude extract was centrifuged at 20,000 g for 20 min at 4°C. The clarified extract was dialyzed overnight against phosphate buffered saline, pH 7.5 at 4°C. Total protein concentration of the extract was determined using the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, Ill.).

ELISA for Anti-Cry9c IgE

The development of the ELISA was initially done by matrix titration of purified recombinant Cry9c. The optimal concentration range of r-Cry9c to be used for coating ELISA plates was determined by maximizing signal to background of immune versus normal goat serum. Antigen concentrations of as high as 100 µg/ml and as low as 0.049 µg/ml were tested. Purified r-Cry9c solution was suspended to the desired concentration in 0.15 M carbonate/bicarbonate buffer, pH 9.6. One hundred microliters of the appropriate antigen dilution was added to wells of an Immulon I ELISA plate (Dynex Technologies, Chantilly, Va., USA) and the plates coated at 4°C for 18–24 h. Plates were then washed with PBS and blocked by incubation with 100 µl PBS containing 10% heat inactivated fetal bovine serum (HIFBS) at room temperature for 2 h. The blocking solution was then aspirated and the plates washed 2 times with PBS prior to use in the ELISA.

IgE ELISA for Control Antigens

Matrix titrations were performed for cat, rye grass and peanut extracts by optimizing the signal to background ratio of randomly selected normal human serum versus serum from patients with known allergies to the various food and environmental allergens. The procedure was similar to that used for r-Cry9c with respect to reagents used for coating, blocking and washing of plates.

ELISA Procedure for Adverse Event and Control Sera

Based on matrix titrations, the concentration of r-Cry9c used for coating test plates was 2 µg/ml. This concentration was at the upper end of the optimal range with respect to the quantity of antigen that could be used for plate coating without a significant increase in normal serum background. Cat, rye grass and peanut extracts were used at 40 µg/ml. ELISA plates were configured so that in addition to r-Cry9c, 16 wells of each plate were coated with cat, rye grass or peanut extract. All sera were diluted 1:2 in diluent buffer (PBS-5% HIFBS, 0.05% Tween 20) and 100 µl was added to duplicate wells and incubated at 4°C overnight (18 h). Adverse event, normal sera, and additional control sera were tested against r-Cry9c. Sera from cat, rye grass, or peanut allergic individuals were included on every plate and tested against the appropriate homologous extracts. R-Cry9c-Immune and normal goat serum (1:5,000 dilution) were also included on each plate in r-Cry9c coated wells. After overnight incubations, plates were washed 4 times with PBS-0.1% Tween 20 (wash buffer). After washing, 100 µl of a 1:2,000 dilution of peroxidase-conjugated affinity purified goat anti-human IgE (KPL, Gaithersburg, Md., USA) was added to each well that contained human serum. Control wells that contained goat serum received a 1:2,000 dilution of peroxidase-conjugated donkey anti-goat IgG (Jackson ImmunoResearch, West Grove, Pa., USA). Plates were then incubated for 2 h at room temperature, washed 4 times with washing buffer, and developed by

addition of 100 µl/well TMB-Elisa substrate-indicator solution (Gibco) for 15 min at room temperature. Color development was stopped by addition of 100 µl/well 1 N H₂SO₄, and the plates read on an ELISA plate reader based on absorbance at 450 nm.

Results

SDS-PAGE analysis of r-Cry9c revealed 2 protein bands of approximately 66 and 56 kD MW (fig. 1). Both bands reacted strongly with goat antiserum to rCry9c in Western blot. The antiserum also reacted with a band of similar MW to the 66-kD band that was present in Starlink corn flour extract. No reaction was seen with an extract prepared from corn flour containing no Starlink. Duplicate blots treated with control normal goat serum were negative. Because the 56-kD band was not consistent with the calculated MW of r-Cry9c, the identity of the 2 bands from the r-Cry9c gel was confirmed by electrospray mass spectrometry and partial sequencing. Data from this analysis indicated that tryptic peptide fragments from both bands were consistent with sequence data from Cry9c (accession No. CAC36454) suggesting that the 56-kD band is an N-terminal truncated form of the larger molecule.

In the first 2 runs, a total of 17 adverse event sera were tested by ELISA for IgE directed against r-Cry9c. In addition, sera from 21 randomly selected EIS officers were included as negative controls. Two types of positive controls were included on each ELISA plate: sera from individuals with clinical allergy to inhalation antigens (cat and grass) or a food allergen (peanut), and a goat antiserum produced against r-Cry9c. Other controls included sera containing either high or low levels of total IgE that were used to assess the effects of high levels of total IgE on the specificity and background of the ELISA. All sera were tested in duplicate at a 1:2 dilution. Reagent blank wells containing only the dilution buffer were also included on each plate. Results of the ELISA for adverse event sera, negative and atopic controls are shown in figure 2. The data presented represent the results from 2 runs in which the original 17 adverse event were tested. An additional run was performed that included 1 additional control and 1 additional adverse event serum as well as several repeat negative control and atopic sera. Using standard protocol [7], a positive (reactive) ELISA test was defined by an absorbance reading that exceeds the cut-off value computed by multiplying the run-specific mean of the negative control group by 2.5. These cut-off values were 0.245, 0.195, and 0.428, respectively, for the three test runs. The highest absorbance reading for the adverse event cases

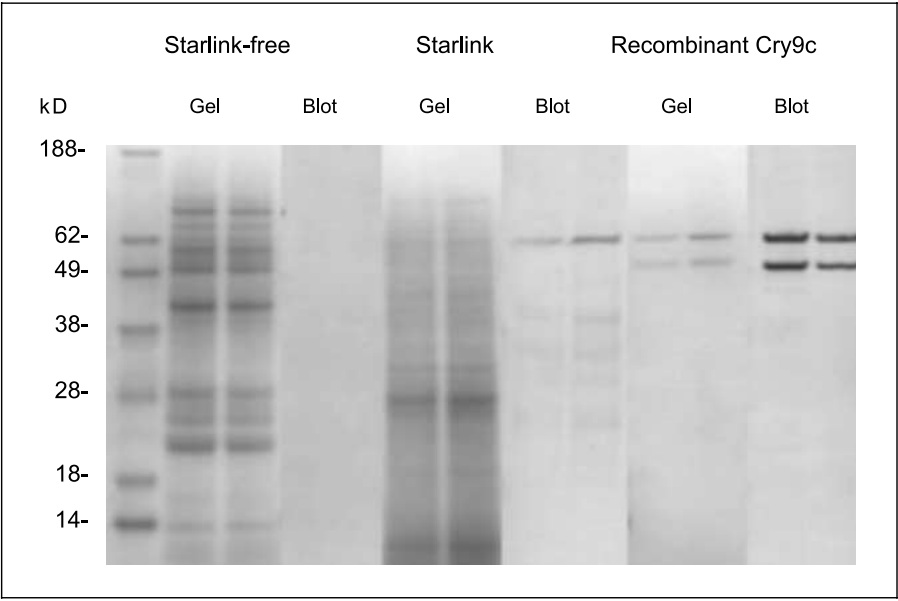


Fig. 1. SDS-PAGE and Western blot analysis of recombinant Cry9c, corn flour prepared from Starlink, and corn flour prepared from non-Starlink corn.

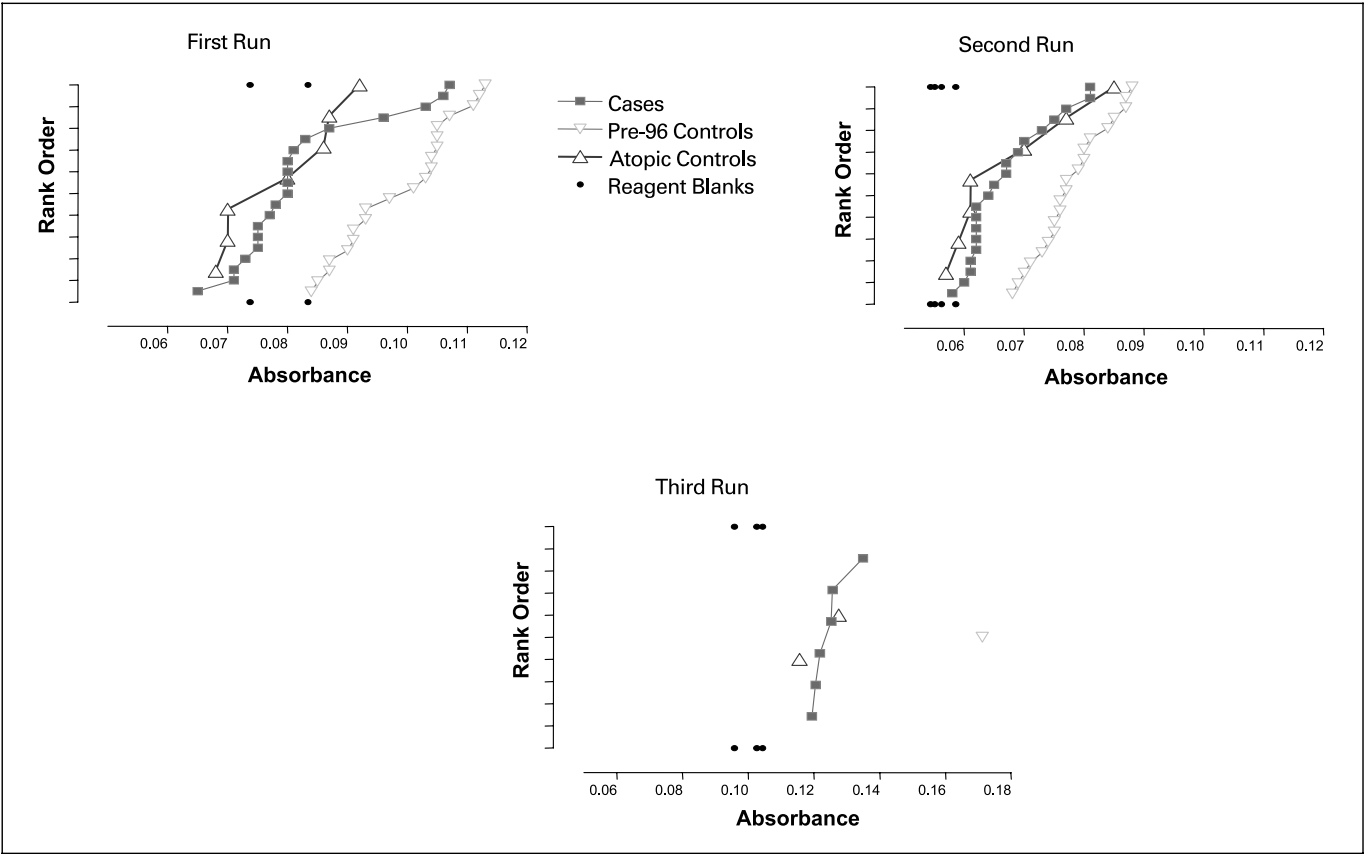
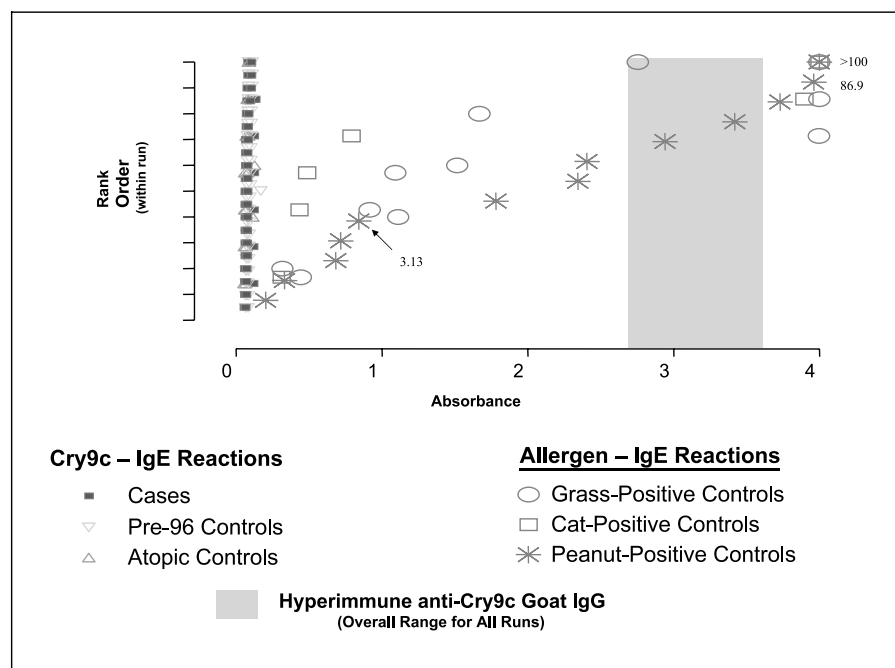


Fig. 2. Results from 3 independent runs of the IgE-specific Cry9c ELISA.

Fig. 3. Comparison of the reaction of adverse event, normal control and allergen-specific sera in IgE-specific ELISA with Cry9c or homologous allergens. Numbers indicate levels of specific IgE (kIU/l) for peanut.



was 0.107 for the first run, 0.081 for the second run, and 0.136 for the third run, each of which are considerably lower than their respective cut-off values. If the cut-off values are re-calculated using the atopic mean values (high total IgE) as our control reference, none of the case serum specimens exceed the cut-off values computed from the first run (0.198), the second run (0.168), or the third run (0.305). The reactivity of all serum samples with Cry9c when assessed by direct comparison with the reagent blank, which contained no serum at all was considered non-reactive if it produced an absorbance reading less than twice the average reading of the reagent blank. All adverse event sera were non-reactive. Various sera from cat, grass and peanut were included in both runs, although the same sera were not always included in all runs. In order to more clearly compare adverse event and other negative controls, the data from 2 runs were shown on an expanded absorbance scale that accommodated all absorbance readings (fig. 3). Adverse event, pre-1996 and atopic sera are compressed against the origin. Sera from allergic patients, tested against their homologous antigen showed a broad range of values, all of which were classified as reactive, based on the above criteria.

Discussion

Our ELISA method was designed only to detect IgE serum antibodies that reacted with Cry9c. It is possible that other antibody isotypes reactive with Cry9c were present; however, other classes of antibody are not definitively associated with immediate hypersensitivity reactions [8, 9]. It is also possible that IgE antibodies were present in such low levels in serum samples from case subjects that the ELISA could not detect them. Other methods of detection such as the radioallergosorbent test (RAST), or the CAP™ [10] test may be more sensitive but are technically more difficult to develop under time constraints. It is also possible for people to have food allergies without any detectable serum IgE to the allergen if most IgE is fixed on tissue mast cells and basophils, or if the response is T-cell mediated [11, 12]. Other studies have demonstrated the presence of IgE and IgG antibodies to extracts of *B. thuringiensis* spores and vegetative organisms in farm workers exposed by inhalation [13]. It was not determined whether any IgE antibodies were directed against Cry proteins present in the extracts.

Because the control goat antiserum was prepared by immunization with r-Cry9c preparations similar to the antigen preparation, reactivity on Western blots with the gel bands would not be conclusive proof that the Cry9c antigen preparation contained only Cry9c protein or that

the antiserum was specific for Cry9c alone. One source of extraneous protein contamination might be bacterial proteins remaining after purification. Confirmation by mass spectrometer analysis that the only two bands present in the SDS-PAGE gel of the antigen preparation were both consistent with r-Cry9c eliminated the possibility that extraneous proteins were present. With respect to the antibody, reactivity with other Cry proteins was not determined. The occurrence of cross-reactivity was not relevant to the purpose for which the antibody was used, which was to verify and optimize binding of the r-Cry9c to ELISA plates. Cross-reactivity with other groups of *B. thuringiensis* pesticidal crystal proteins (e.g., Cry1, Cry 3) would be unlikely. Based on phylogenetic classification and nomenclature conventions, such distinct groups are no more than 45% homologous [14]. It is also of interest that the goat antiserum reacted with a protein of indistinguishable MW in corn flour prepared from Starlink corn, but not with flour made from corn harvested prior to the introduction of Starlink. This indicates some antigenic similarity between corn and bacterially produced Cry9c. It also suggests that the degree of glycosylation of Cry9c, as expressed in Starlink, is not sufficient to drastically alter its gel migration pattern.

We did not have a positive human serum control that reacted with Cry9c. Indeed, it is probable that in any scenario in which possible allergic reactions to a novel food protein are reported, no such serum will be readily available. The approach used to circumvent this problem was to incorporate controls for as many aspects of the ELISA as possible. Accordingly, the coating of ELISA plates with r-Cry9 was confirmed by binding of goat anti-Cry9c antiserum. In order to verify the ability of the anti-IgE detection reagent to detect bound human IgE, ELISA procedures were developed to test for IgE antibodies to known environmental and food allergens. These ELISA methods were capable of detecting IgE antibodies to other allergens (cat, grass, and peanut) in every control sample tested. In the case of peanut, some specific IgE levels were known, and encompassed a fairly broad range. In this case, the sample with the lowest measured specific IgE level was distinctly positive. This suggests that the Cry9c ELISA would likely have been able to detect IgE antibodies, had they been present in serum at levels comparable to those seen in other allergic individuals. It is possible that glycosylation of Cry9c as it is expressed in Starlink corn might affect reactivity if any IgE epitopes were to involve carbohydrate moieties, since such epitopes would not be present in bacterially expressed r-Cry9c. SDS-PAGE/Western blot analysis suggests that large differences in

MW do not exist between corn and bacterially expressed Cry9c, however some glycosylation cannot be ruled out, and it remains possible that IgE directed against a carbohydrate containing epitope could have gone undetected in the ELISA.

The accidental release of Starlink corn presented a number of problems related to developing methods intended to produce evidence of exposure or reactivity in individuals with possible exposure. These problems might also be encountered in a similar event in the future. Thus, this episode and the response to it may serve as a guide for a future response. The focus on IgE-specific antibodies is valid because of their demonstrated role in clinical allergy; however, the presence of serum IgE alone may not correlate with clinical allergy. In addition, detection of IgG antibodies might provide evidence of exposure, but would not correlate with allergic reactions. The presence of specific serum IgE in this, and any future, study would provide participants with information that they could then use to seek further guidance from their health care provider to further define their personal allergy potential. With respect to protein standards, it would be useful if the producers of bioengineered products maintained sufficient stocks of the purified protein for the development of ELISA tests or other detection methods. Ideally, a quantity of the protein should also be available in its plant-produced form. As was the case here, availability of a hyper-immune antiserum would also facilitate assay development. It is also assumed that producers can serve as a source of information on the molecular characteristics of proteins such as sequence data and especially with respect to glycosylation patterns in the plant host.

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