June 11, 2001

Investigation of Human Health Effects Associated with Potential Exposure to Genetically Modified Corn

A Report
to the U.S. Food and Drug Administration
from the Centers for Disease Control and Prevention

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Executive Summary

On October 25, 2000, the U.S. Food and Drug Administration (FDA) requested technical assistance from the Centers for Disease Control and Prevention (CDC) in investigating adverse event reports (AERs) of human illnesses that were potentially associated with consumption of genetically modified corn products. Prior to these reports, a protein named Cry9c had been inserted into genetically modified StarLink™ corn; it subsequently and inadvertently was introduced into the human food supply. CDC conducted an epidemiological investigation that included (1) reviewing the AERs, (2) administering questionnaires to all people who experienced adverse health effects and manifested signs and symptoms consistent with allergic reaction, (3) obtaining relevant medical records, and (4) collecting serum samples for temporary banking. The investigation concluded that 28 people had experienced apparent allergic reactions. These people had also reported eating corn products that may have contained Cry9c protein. With the endorsement of U.S. Environmental Protection Agency's Scientific Advisory Panel which convened on November 28, 2000, CDC recommended that the banked serum samples be evaluated to see if they contained evidence of an allergic response to the Cry9c protein.

An FDA laboratory developed an enzyme-linked immunosorbent assay (ELISA) method to detect antibodies to the Cry9c protein. CDC sent coded serum samples to FDA for analysis, including serum samples from the affected people and historically banked serum samples collected before Cry9c entered the food supply. CDC also sent serum samples from people identified as being highly sensitive to a variety of allergens. The ELISA method found that none of the CDC-submitted samples reacted in a manner consistent with an allergic response to the Cry9c protein.

These findings do not provide any evidence that the reactions that the affected people experienced were associated with hypersensitivity to the Cry9c protein. The difficulties of this investigation highlight the importance of evaluating the allergic potential of genetically modified foods before they become available for human consumption.

Investigation of Human Health Effects Associated with Potential Exposure to Genetically Modified Corn

A Report
to the U.S. Food and Drug Administration
from the Centers for Disease Control and Prevention

This report presents the results of an epidemiological investigation and a research study that the Centers for Disease Control and Prevention (CDC) conducted at the request of the U.S. Food and Drug Administration (FDA). CDC conducted this work to assess potential public health hazards from the inadvertent release of genetically modified corn into the human food supply. The focus of this study was to evaluate the potential for allergic reactions among consumers of corn-containing food products.

Background

StarLink™ corn (Aventis Crop Science USA LP) contains the protein Cry9c, genetically modified from the *Bacillus thuringiensis* subspecies *tolworthi* bacteria. This protein has pesticidal properties and was genetically inserted into StarLink™ corn to protect the crop against several insects, including the European corn borer, the cornstalk borer, and the corn earworm. In May 1998, the U.S. Environmental Protection Agency (EPA) granted a limited license for the production of StarLink™ corn. The license proscribed that this corn variety was to be grown only for animal feed, industrial nonfood uses, and seed increase. EPA did not license StarLink™ corn for use in food intended for human consumption because the Cry9c protein shared several molecular properties with proteins that are known food allergens. Despite the EPA ruling, Cry9c-DNA was detected in taco shells in September 2000. This discovery caused several food distributors to recall implicated product lines. Following the media coverage of the food product recalls, FDA began receiving reports of adverse health events from consumers who had eaten food products containing corn. In addition, the U.S. Department of Agriculture (USDA) pointed out that all corn, including the StarLink™ corn variety, is, in common practice extensively comingled after harvest. Ultimately, it was not known how much of the genetically engineered corn had entered the human food chain.

On October 25, 2000, FDA invited CDC to help review and investigate related adverse event reports (AERs) submitted to FDA. Further discussions were held between representatives of CDC's National Center for Environmental Health (NCEH), FDA, EPA, and USDA. On November 16, 2000, CDC assigned EPI-AID #2001-13 to the Health Studies Branch, Division of Environmental Hazards and Health Effects, NCEH, CDC, to conduct the initial review and investigation.

Epidemiological Investigation

Since 1946, CDC has used a mechanism called EPI-AID to provide rapid epidemiological response to investigate potential threats to public health. The objectives of EPI-AID #2001-13 included the following:

1. To quickly determine if FDA AERs were consistent with allergic reactions to food;
2. To obtain serum samples from individuals in this study who met the case definition for allergic reactions after eating corn-containing food (for possible future serologic testing);
3. To determine if development of a Cry9c-specific serologic test was warranted.

FDA personnel performed an initial triage of all AERs received since July 1, 2000, selecting those that included consumption of corn products. FDA provided blinded copies of these reports to CDC for review. CDC reviewed the AERs and developed a working case definition for further investigation.

A case was defined as a report forwarded to CDC concerning human consumption of a corn product occurring between July 1 and November 30, 2000, that manifested as follows:

- A suspected anaphylactic reaction (e.g., dizziness, weakness, or loss of consciousness) that occurred within 1 hour of product consumption, or
- Any of the following dermatological or oropharyngeal symptoms (hives, rash, pruritis, oropharyngeal tingling or swelling) that occurred within 12 hours of product consumption, or
- Any of the following gastrointestinal symptoms (vomiting, diarrhea, abdominal cramping) that occurred within 12 hours of product consumption and that involved only one individual among meal companions, and
- These symptoms not explained by a pre-existing medical condition

FDA requested permission from all reporting individuals to release their identifying information so that CDC could directly contact individuals who had filed AERs. CDC interviewed individuals, either by phone or in person, using a questionnaire that incorporated a validated food allergy survey. CDC also invited each person to provide a blood sample that was to be banked pending the outcome of the CDC investigation and in anticipation of developing a Cry9c-specific serologic test. CDC obtained informed consent from each individual (see Appendix A).

CDC received AERs from FDA involving 51 individuals. Of the 51 individuals who experienced adverse health effects, 23 did not meet the CDC case definition for the following reasons: four experienced symptoms other than those included in the case definition; five had symptoms that did not occur within the established time frame following product consumption; two had symptoms that were attributed to a previously diagnosed illness; and 12 had meal companions who also experienced gastrointestinal symptoms, suggesting infectious causes of foodborne

illness.

Of the 28 individuals meeting the case definition, 25 gave FDA permission to release identifying information to CDC; however, one individual who gave permission to be contacted never responded when contact was attempted. CDC completed interviews with 24 of the people who filed AERs and who also met the CDC case definition. These 24 case subjects ranged in age from 5 years to 74 years, with a mean age of 36 years; 13 cases were male and 11 were female. They resided in 15 states (California, Florida, Georgia, Illinois, Kansas, Maryland, Massachusetts, Missouri, New Jersey, North Carolina, Ohio, Texas, Virginia, Washington, and Wisconsin), the District of Columbia, and the Commonwealth of Puerto Rico (see Figure 1). No more than 2 people were from the same state or territory. Seventeen of the people who completed questionnaires also provided serum samples.

For 10 of the 24 case subjects, symptom onset was rapid (within 1 hour), and most people reported multiple symptoms. One individual reported loss of consciousness, and two others reported weakness or dizziness, within 1 hour of product consumption. Nineteen individuals sought medical care, and 19 of the 24 case subjects were either self- or physician-treated for allergic reaction. Two people were hospitalized. Case individuals reported having eaten several different corn products from a variety of manufacturers, including corn taco shells, corn tortillas, corn chips, corn cereal, wheat flour tortillas, black beans and rice, and breaded chicken nuggets. (The last three foods are not technically considered corn products, but each listed corn as an ingredient).

CDC presented preliminary results of the epidemiologic field investigation to an EPA scientific advisory panel (SAP) on November 28, 2000. The preliminary findings suggested the utility of developing an enzyme-linked immunosorbent assay (ELISA) to further assess the relation between allergic manifestations and the Cry9c protein. SAP endorsed this recommendation, and CDC began to develop a research protocol to assess the case serum samples as well as comparison serum samples.

Research Protocol

In response to the findings of the EPI-AID investigation, CDC developed a protocol (IRB approval #3019) for collecting serum from groups of people with the potential for being differentially exposed to Cry9c. Simultaneously, FDA laboratories developed a test method for detecting Cry9c-specific IgE antibodies in serum. 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.

Methods

For this study, CDC secured serum samples from three distinct groups of people: people who reported the adverse health effects, people identified as being highly sensitive to a variety of allergens, and people who had historically banked serum samples collected before Cry9c entered the food supply. CDC sent these as coded serum samples without any personal identifiers or category identifiers to FDA for analysis. After consultation with scientists and physicians at other federal agencies, CDC decided to request that serum specimens be tested only for IgE reactivity with Cry9c. Since IgE is the only type of antibody that causes immediate hypersensitivity in humans, any other antibody reactivity (IgG or IgA) would be irrelevant to the immediate-type allergic reactions specified in the case definition. All serum specimens in this study were tested using the Cry9c-specific-IgE-ELISA method (Cry9c-ELISA) that FDA developed.

SERUM GROUP A

This group of 17 samples was from the people who provided serum as part of EPI-AID #2001-13, described above. These people had contacted FDA to report an adverse health event after consuming food products containing corn and met the case definition set by CDC to be included in this study.

SERUM GROUP B

This group of serum samples was from six people with documented multiple allergies. These people are referred to as "atopic" because they have high levels of IgE circulating in their serum. An atopic person is more likely than the average person to have an immune response if exposed to an allergenic ingredient in food. Investigations by both the USDA and FDA revealed that Cry9c was widely dispersed in corn supplies in small amounts. Serum from Group B was used to assess for reactivity against Cry9c. A positive test result among serum samples in Group B might suggest that either the Cry9c protein was allergenic or that there was nonspecific IgE binding in this new assay (a lack of specificity).

SERUM GROUP C

Group C consisted of 21 banked serum samples drawn prior to1996 (before StarLink™ corn was ever grown) from Epidemic Intelligence Service Officers at CDC. Serum from Group C was used to verify the absence of cross-reactivity with proteins that were already in the food supply before Cry9c was released. This group was determined, a priori, to be the comparison group ("negative control") for Group A ("cases").

To avoid bias in the laboratory analysis, CDC sent serum samples from Groups A, B, and C to FDA as a batch (except for 1 case and 1 atopic control which were late arrivals to CDC), and labeled the specimens with just a simple code number. CDC sent a second batch to FDA containing the last case and atopic control serum specimens, along with replicas of cases previously sent to avoid analytical bias. FDA tested the batches on separate days using the new

Cry9c-ELISA (see Appendix B, FDA Procedure for Detection of Antibodies to Cry9c). All specimens in the first batch were tested at the same time within the same analytical run on two different days, along with internal controls. The same was done for the second batch. Internal controls included a reagent blank (wells to which sample diluent without serum was added) and goat serum both from normal goats (nonimmunized) and from goats that were purposefully sensitized against the Cry9c protein ("positive control" for the assay). Wells coated with cat, grass, or peanut allergen were also used to ensure performance of the assay. Serum from people known to be allergic to cat, grass, or peanuts were applied to wells coated with the respective allergen to assess if the assay was able to detect allergen-specific IgE. The results consisted of optical density values that define the level of light absorbance in a specific well. The value of a specific coded sample cannot be directly compared to a second run of that sample; all of the values in a particular run are interpreted with regard to their relation to each other and to the blank samples and the positive serum samples from sensitized goats.

Interpretation of ELISA Results

A priori, we determined that serum banked before the release of StarLink™ corn (**GROUP C**) comprised the negative controls for comparison with the cases (**GROUP A**). Serum from multiple allergic, or atopic, individuals (**GROUP B**) were tested to ensure that the presence of high IgE did not give a false-positive test result.

Figure 2 shows the distribution of absorbance values that were documented in each group during the first FDA run. Duplicate samples were included for internal quality control and account for additional data points on the plots. There is expected variation around the run-specific diluent blank. For the 16 cases in **GROUP A**, the absorbance readings are between 0.06 to 0.11. For the five atopic samples in **GROUP B**, the absorbance readings are between 0.06 to 0.09, and for the 21 pre-1996 samples in **GROUP C**, the absorbance readings are between 0.08 to 0.12. Figure 3 shows the same information for the second FDA run. The first run had a slightly higher reagent blank absorbance reading (0.07-0.10) than in the second run (0.05-0.06). The first run also showed greater overall variance in the absorbances in cases and a priori controls than did the second run. In both runs all cases have lower absorbance readings than the pre-1996 controls (**GROUP C**). Figure 4 displays the third FDA run, which includes an additional case sample that arrived later than the other samples as well as several duplicates of previously run samples.

In all three runs, the readings from stored pre-96 controls are generally higher than the readings from freshly drawn serum (cases and atopic controls). It is not uncommon to see higher background absorbance readings in serum samples frozen and stored for longer periods (**GROUP C** or pre-1996 controls) than in fresher serum samples (**GROUP B** or atopic controls; Oliver 2000). There are no other consistent trends among the replicated serum specimens. The absorbance readings for all groups appear to reflect variability in the background range.

Using standard protocol (Rose 1992), 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 **GROUP C** (determined a priori to be the negative control) by 2.5, as shown below.

Calculation:	
First run	2.5 (0.098) = 0.245 A reactive ELISA test must exceed the absorbance reading of 0.245 for the first run.
Second run	2.5 (0.078) = 0.195 A reactive ELISA test must exceed the absorbance reading of 0.195 for the second run.
Third run	2.5 (0.171) = 0.428 A reactive ELISA test must exceed the absorbance reading of 0.428 for the third run.

The highest absorbance reading for the cases (**GROUP A**) 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 calculated from **GROUP C** (i.e., 0.245, 0.195, and 0.428). Even if we recalculate the cut-off values using the atopic (**GROUP B**) mean values 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 or with control allergens (cat, grass, peanut) was also assessed by direct comparison with the reagent blank, which contained no serum at all. Any serum that produced an absorbance (optical density) reading less than twice the average reading of the reagent blank was considered "non-reactive", any serum that produced an absorbance reading greater than ten times the average reading of the reagent blank was considered "strongly reactive," and any serum that produced an absorbance reading between those ranges was considered "reactive." These results are summarized in Table 1.

Figure 5 demonstrates that the positive signal obtained with the Cry9c-immunized goat serum is three to four orders of magnitude greater than any of the human serum samples (**GROUPS A, B, and C**). This suggests that the ELISA was sufficiently sensitive to detect low concentrations of Cry9c antibody.

Figure 5 also shows the results that FDA reported of other serum samples from people with known allergies (i.e., cat, grass and peanut) that were analyzed at the same time as the CDC samples in this study. Using the same ELISA with cat, grass, and peanut allergens, FDA was able to accurately detect antibodies to the known substances causing allergies in these people. This finding serves as an additional internal quality control procedure.

We found very similar patterns when we reviewed the results that a University of Maryland laboratory obtained when they analyzed the same set of samples that CDC sent to FDA.

Limitations

Our analysis was designed only to detect IgE antibodies that reacted with Cry9c, and we did not have a positive human serum control that reacted with Cry9c. It is possible that other antibodies to Cry9c were present, or that IgE antibodies were present in such lows levels in serum samples from case subjects that the ELISA could not detect them. It is also possible for people to have food allergies without any detectable IgE to the allergen (Ogura 1993). However, the FDA ELISA method was capable of detecting IgE antibodies to other allergens (cat, grass, and peanut) in every control sample tested, and these results were replicated by an independent laboratory.

Summary and Recommendations

Table 1 summarizes the results of the various samples that were analyzed using the Cry9c IgE-specific ELISA test. This table displays the nonreactivity of all of the human samples to the Cry9c protein, while also showing the ability of the test to react to known allergens and to hyperimmune goat serum. Cry9c-specific-IgE was not detected in any of the human serum specimens using an ELISA that was capable of detecting IgE to other allergens in people with known hypersensitivity to them. This table also points out that there was no positive human control for this test method.

Although the study participants may have experienced allergic reactions, based upon the results of this study alone, we cannot confirm that a reported illness was a food-associated allergic reaction. Although our results do not provide any evidence that the allergic reactions experienced by the people who filed AERs were associated with hypersensitivity to Cry9c protein, we cannot completely rule out this possibility, in part because food allergies may occur without detectable serum IgE to the allergens (Ogura 1993). We recommend that the study participants share the study results with their health care providers.

Evaluating the public health implications from the inadvertent introduction of StarLink™ corn into the human food supply posed a challenging retrospective task. The difficulties of this investigation highlight the importance of evaluating the allergic potential of genetically modified foods before they become available for human consumption.

References

Ogura Y, Ogura H, Zushi N, Morita H, Kurashige T. The usefilness and the limitations of the radioallerosorbent test in diagnosing food allergy in atopic dermatitis. Arerugi - Japanese Journal of Allergology. 1993;42(6):748-56.

Oliver DW, Walker, MS, Walters AE, Chatrath P, Lamberty BG. Anti-silicone antibodies and silicone containing breast implants. British Journal of Plastic Surgery. 2000;53(5):410-4.

Rose NR. Manual of Clinical Laboratory Immunology, 4th ed. 1992:697.

Figure 1. Distribution of Adverse Event Reports Made to FDA That Met CDC Case Definition for Possible Allergic Reaction Following a Meal Containing a Corn Product

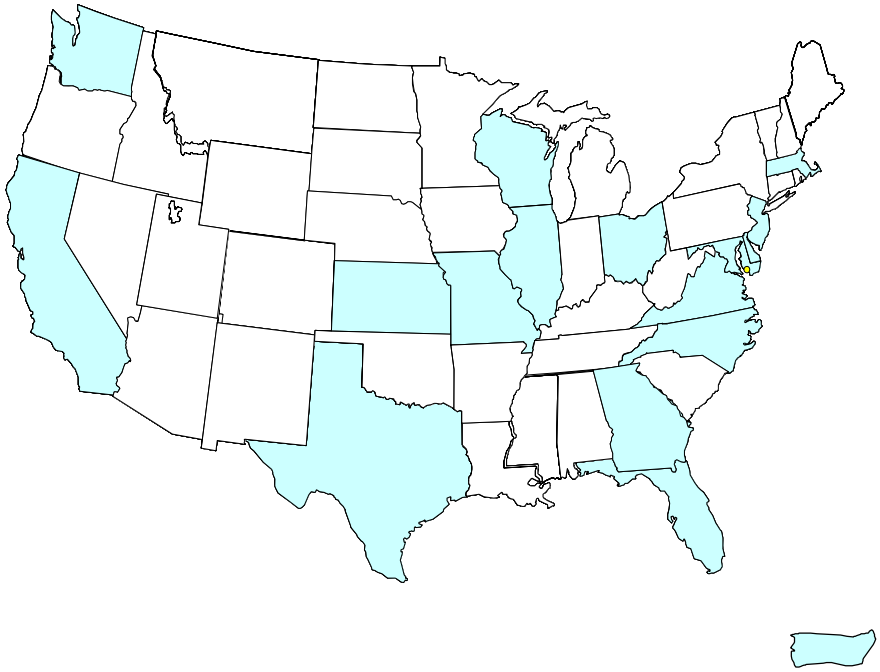

Figure 2. FDA Data First Run

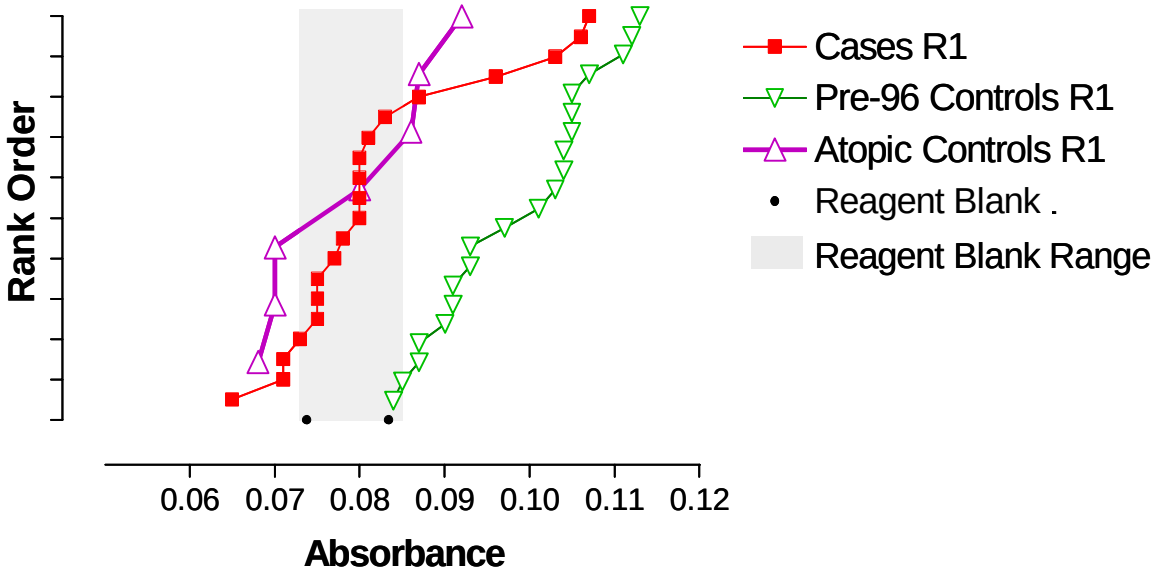

Figure 3. FDA Data Second Run

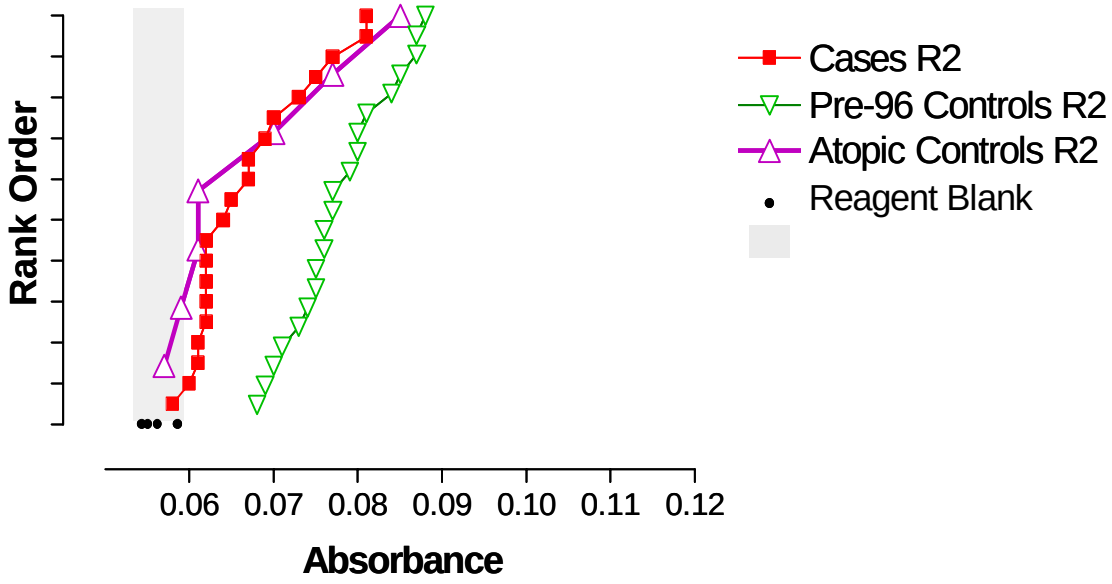

Figure 4. FDA Data Third Run

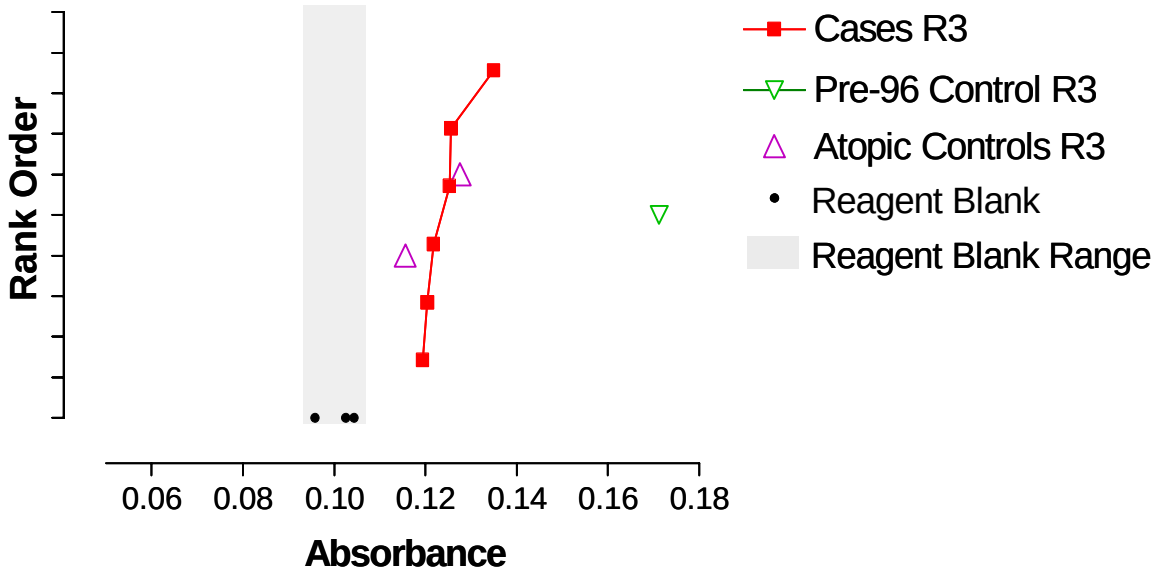

Figure 5. Overall Depiction of FDA ELISA Results: IgE Reactivity to Cry9c in Cases, Pre-1996 Controls, and Atopic Controls and IgE Reactivity to Cat, Grass, and Peanut Allergens in Positive Controls

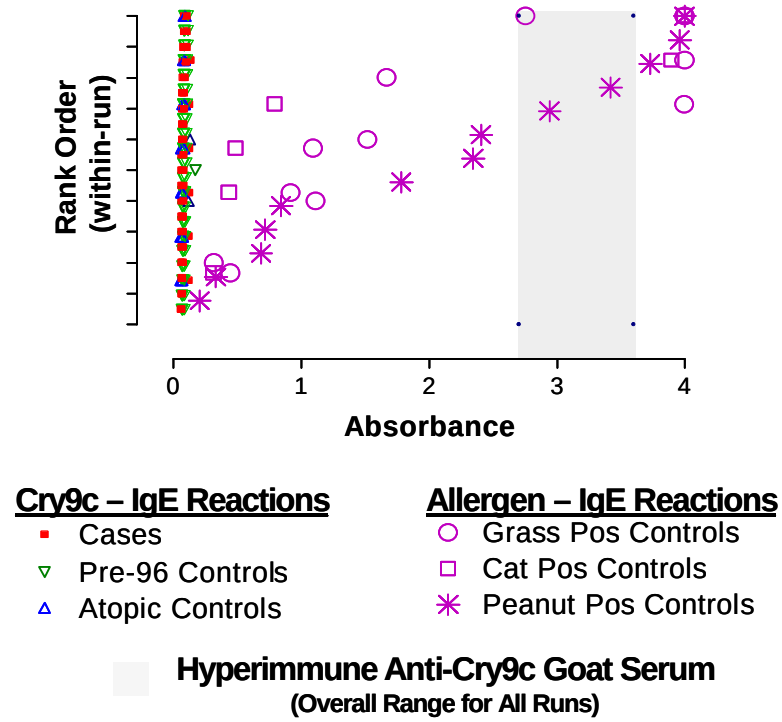

Table 1. Summary of Results

Antigen	Serum Source	Antibody Detected	Results
None	None	Human IgE	Blank
Cry9c	Pre-1996 CDC controls	Human IgE	All nonreactive
Cry9c	Atopic CDC controls	Human IgE	All nonreactive
Cry9c	Epi-Aid cases	Human IgE	All nonreactive
Grass	Positive controls*	Human IgE	All reactive to strongly reactive
Cat	Positive controls*	Human IgE	All reactive to strongly reactive
Peanut	Positive controls*	Human IgE	All reactive to strongly reactive
None	None	Goat IgG	Blank
Cry9c	Normal goat serum	Goat IgG	Nonreactive
Cry9c	Hyperimmune goat serum**	Goat IgG	Strongly reactive

Nonreactive: Less than twofold blank reading
Reactive: Twofold to tenfold blank reading
Strongly reactive: Greater than tenfold blank reading

*Positive controls were obtained from IBT Laboratories, Inc.
**Hyperimmune goat serum was obtained from goats immunized with Cry9c.

Appendix A

CONSENT TO PARTICIPATE

An investigation by the Centers for Disease Control and Prevention to determine
The risk of food allergic reactions from corn products containing Cry9C

The Centers for Disease Control and Prevention is conducting an investigation to gather information from people who reported adverse health events after eating corn products containing the genetically engineered protein, Cry9C. Since you reported an adverse event, we would like you to be part of this investigation. Your participation is completely voluntary. Refusing to participate is your right and there is no penalty for doing so. The choice is yours. Before you decide, you should know what is involved and have all your questions answered.

Purpose
We will interview and collect blood samples from as many of the people who reported adverse reactions as possible. The combined results of the interviews and blood tests will help us better understand whether or not corn products containing Cry9C protein can potentially cause food allergic reactions and what the risk to individuals might be.

What will it involve?
If you agree, we will take a blood sample right now. It should take about 5-10 minutes. We will take the sample (1 or 2 teaspoons) from a vein in your arm. We will label your blood sample with a unique identification number instead of using your name or other personal information.

A new blood test is being considered for development to detect antibodies against the genetically engineered protein Cry9C. Because of the need to quickly gather information from individuals who reported adverse reactions to corn products containing Cry9C, we are asking questions and collecting blood samples now, even though the blood test is not yet available. If the blood test is not available for use by 31 March 2001 all blood samples will be destroyed, including yours.

If the test is developed, your blood will be tested only for the presence of Cry9C antibodies; it will absolutely not be tested for anything else at any time (such as HIV testing, genetic testing, or drug testing). After the Cry9C antibody testing is complete, any left-over blood will be destroyed.

Cost
There is no cost to you.

Benefits
The goal of this investigation is to help answer the question of whether or not corn products containing the genetically engineered protein Cry9C can potentially cause allergic reactions when eaten by people. The information may help determine if this product is safe for use in human foods and this may indirectly benefit you. Also, if the blood test is developed, we will notify you of your test results if you desire to know.

Risks
The only risk is that you may have some mild, brief discomfort when we take blood from your arm. A small bruise may also appear. The individual who will take your blood is trained and experienced and will do everything possible to minimize your discomfort.

Confidentiality
We will keep your test results and what you tell us confidential to the extent allowed by law. We will notify you of your blood test result if you wish to know, and a summary of all the test results will be given to the U.S. Food and Drug Administration. We will not tell anyone else what your test result is.

To protect your privacy, we will not put your name on the questionnaire or blood sample but will use a unique identification number instead. We will also keep all the investigation records and blood sample results in locked files and only investigation staff will be allowed to look at them. When we talk or write about the investigation, we will not include your name or other facts that might identify you.

We are putting your name, telephone number, and address on this consent form now so that we can notify you of your test results and to document your willingness to participate. We will keep this consent

form in a locked file separate from the questionnaires and blood test results.

For more information
We will give you a copy of this form to keep. We would be happy to answer any questions about the study or a problem or injury related to the investigation. You may contact the principal investigators, Dr Brad Winterton and Dr Dori Reissman, at the Centers for Disease Control and Prevention at 404-639-2530. If you have any questions now or in the future about your rights as a participant in this investigation, you may contact the CDC Deputy Associate Director for Science at 800-447-4784, mailbox number 329-4518. Leave a message with your name and number and someone will call you back.

Voluntary participation
It is your choice whether or not to participate in this investigation. There is no penalty if you choose not to participate.

If you agree to volunteer to participate in this investigation, you must sign below. By signing this form, you are saying that:

1. **You have read this entire form or someone read it to you completely.**
2. **All of your questions have been answered.**
3. **You are volunteering to be part of this investigation.**
4. **You know that you have no obligation to answer any questions or to give a blood sample.**
5. **You will be given a copy of this form to keep.**

I have read this informed consent and agree to participate in this investigation.

_____ _____
(Signature) (Date)

_____
(Printed name)

Street Address: _____
_____

City: _____ State: ____ Zip: _____

Phone number: _____

Would you like to be notified of the results of your blood test? YES NO

Parents of children under 18 years old:
As the parent or legal guardian of this person, I give permission for _____ to participate in this investigation.
(Participant's name)

_____ _____
(Parent or guardian signature) (Date)

_____
(Parent or guardian printed name)

Consent to release medical records

We would also like your permission to check with any doctors who have given you medical care related to your adverse reaction or other allergies you may have. To do this, we ask you to sign below and also give us the name and address of the doctor or hospital where these records are located. Even if you do not want to give us permission to review your medical records, you may still participate in this investigation by answering the questionnaire questions and giving a blood sample. **By signing below you agree to allow the doctor or hospital listed to share your medical records with us.**

I have read the above consent to release medical records and agree to allow the doctor/hospital listed below to release my medical records for the purpose of this investigation.

_____ _____
(Signature) (Date)

_____
(Printed name)

Name, address, phone of doctor or hospital

_____

_____

_____

_____

Name, address, phone of doctor or hospital

_____

_____

_____

_____

CHILD ASSENT TO PARTICIPATE

An investigation by the Centers for Disease Control and Prevention to determine

The risk of food allergic reactions from corn products containing Cry9C

You may have heard that the government has recalled some foods that contain corn. That is because there is a concern about whether or not those foods are safe to eat. They contain a new type of corn that is genetically engineered so that insects can't eat it.

We are doing an investigation to find out information about people who have eaten some of those foods. We would like you to be in this investigation. You don't have to unless you want to. It is up to you.

What will happen?
If you let us, we will take a small amount of blood from a vein in your arm by putting a needle in the vein for a few seconds. First we will rub your skin with alcohol to clean it.

We will also ask you or your parents some questions about your health and things you might have eaten. Your answers are private. We will not tell anyone else outside the investigation.

Will it hurt?
The needle stick in your skin may hurt a little for a few seconds. The person taking the blood will be very careful.

Benefits
We are doing this investigation to help answer some important questions.

We want you to sign your name on this paper to say that you agree to have the blood test done and answer our questions.

1. **You know that the blood test is just for this investigation.**
2. **You know how we will take your blood sample.**
3. **You know that you do not have to be in this study or answer our questions or have this blood test done if you do not want to.**

_____ _____
(Child's signature) (Date)

_____
(Child's printed name)

_____ _____
(Parent or guardian signature) (Date)

_____
(Parent or guardian name)

Unique Identifier

Street Address: _____
_____

City: _____
State: _____ Zip:_____

Phone number: _____

Appendix B

FDA PROCEDURE FOR DETECTION OF ANTIBODIES TO CRY9C

COAT ELISA PLATES

1. Suspend purified Cry9C solution to a concentration of 2 ug/ml in carbonate/bicarbonate buffer, pH 9.6. Suspend crude grass antigen to a concentration of 40 ug/well, and crude cat antigen to a concentration of 0.2 units/ml. (Optimum concentration of antigen previously determined by block titration with known positive and negative sera.)

2. Pipette 100 ul/well into Dynatech Immulon I plates. Include grass and cat antigen to serve as reagent controls.

3. Incubate overnight at 4C.

ADD SERA TO PLATES

4. Allow plates to equilibrate to RT.

5. Aspirate liquid from wells with 12-channel manifold. Wash plates 1 x with PBS.

6. Block for two hours RT with PBS-10% HIFBS, 100 ul/well.

7. Aspirate liquid from wells as above. Wash plates 2 x with PBS.

8. Dilute sera 1:2 with sample diluent (PBS-5% HIFBS, 0.05% Tween 20). Dilute goat antisera 1:5000 with sample diluent.

9. Add diluted sera to wells, 100 ul/well, in duplicate. Pipette know positive sera in cat and grass antigen-coated wells.

10. Incubate plats at RT for two hours or overnight at 4C.

ADD CONJUGATE

11. Allow plates to equilibrate to RT (if incubated at 4C).

12. Aspirate liquid from wells. Wash plates 4X with PBS-0.1% Tween-20. Allow wash buffer to remain in wells for 1 min.

13. Add affinity purified peroxidase-conjugated goat anti-human IgE (KPL, Cat #074-1002) to wells, 100 ul/well. For wells with goat serum, add affinity purified peroxidase-conjugated donkey anti-goat IgG (Jackson Labs., Cat. #705-035-147). Appropriate dilution of conjugate must be determined for each new lot. Conjugate is diluted in PBS-10% HIFBS.

14. Incubate 2 hrs. at RT.

DEVELOP COLOR

15. Aspirate liquid from wells and wash 4x as above with PBS-0.1% Tween-20.

16. Add 100 ul/well of substrate solution (TMB-Elisa, Gibco Labs., Cat. #15980-014).

17. Incubate 15 min at RT.

18. Stop reaction with 100ul/well 1N H_2SO_4.

19. Read absorbance with 96-well Elisa plate reader, 450 nm.