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Confirmation of a predicted lack of IgE binding to Cry3Bb1 from genetically modified (GM) crops

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ABSTRACT

Some GM crops including MON863 corn and stack varieties contain Cry3Bb1 protein. Cry3Bb1 is very important from the standpoint of assessing the safety of GM crops. In this study Cry3Bb1 was assessed from the standpoint of possible binding to IgE from allergy patients. First, an ELISA that was improved in our laboratory was used to test serum samples from 13 corn allergy patients in the United States with recombinant Cry3Bb1 expressed in *Escherichia coli*, and serum samples from 55 patients in Japan with various food allergies were also assayed. Two samples from the Japanese allergy patients were suspected of being positive, but Western blotting analysis with purified Cry3Bb1 indicated that the binding between IgE and Cry3Bb1 was nonspecific. Ultimately, no specific binding between IgE and recombinant Cry3Bb1 was detected. Next, all proteins extracted from MON863 corn and non-GM corn were probed with IgE antibodies in serum samples from the corn allergy patients by Western blotting, but the staining patterns of MON863 and non-GM corn were similar, meaning that unintended allergic reactions to MON863 are unlikely to occur. Our study provides additional information that confirms the predicted lack of IgE binding to Cry3Bb1 in people with existing food allergies.

1. Introduction

Cry3Bb1 is an insecticidal protein produced by *Bacillus thuringiensis* that kills corn rootworm larvae. The Cry3Bb1 coding sequence was introduced into the corn genome by Monsanto to develop MON863 corn, a grain that is protected against feeding damage by the corn rootworm. Several stack varieties containing the Cry3Bb1 gene were also developed to simultaneously protect corn from different species of insects.

There has been controversy about the safety of GM crops. International regulatory authorities consider it necessary to demonstrate that new crops developed by applying biotechnology methods are as safe as conventional crops, and they have published Codex guidelines (2003) for pre-market research on GM crops. The Codex guidelines have adopted a weight-of-evidence approach, and there are five key elements in the assessment. The first is whether the source of the gene is an organism that is a frequent cause of allergic reactions. The second element is a bioinformatic element, which consists of sequence searches for matches with >35% identity over 80 amino acids (or of >50% overall identity for more realistic risks) between the protein of interest and all known allergens. The third is IgE testing to determine whether IgE antibodies bind the introduced protein. The fourth element is stability testing to determine whether the protein expressed is highly resistant to digestion by pepsin, and the fifth is the abundance and stability of the protein in the food.

There is no international agreement in regard to post-marketing studies of GM crops. The authors think that surveillance studies of the allergenicity of GM crops are needed in order to achieve better acceptance of GM crops. Some people think that the pre-market study of Cry3Bb1 was sufficient and that our study was unnecessary. However, we think it is meaningful to provide as many data concerning the safety of GM crops as possible. This study is one of a series of studies we have been conducting to confirm the predicted lack of IgE binding to novel proteins in genetically modified crops. We performed an IgE binding test of recombinant Cry3Bb1 and an extract of MON863 as surveillance research. However, *B. thuringiensis*, which biosynthesizes Cry3Bb1, does not usually induce allergic reactions, and the Cry3Bb1 sequence does not match that of any known allergens. Moreover, Cry3Bb1 is rapidly and extensively degraded by pepsin. That means that IgE binding tests are not obligatory according to the Codex guidelines. Based on the international criteria, our study was optional. However, it adds information regarding the safety assessment of GM corn containing Cry3Bb1.

In this study, we assessed the binding characteristics to recombinant Cry3Bb1 of IgE antibodies in serum samples of allergy patients. An ELISA that was improved in our laboratory was used to conduct the tests, and the results showed that the IgE antibodies

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in the serum samples of corn allergy patients in the United States and patients with various food allergies in Japan did not specifically bind to recombinant Cry3Bb1.

IgE antibodies from corn allergy patients were also tested for their binding profiles to all proteins extracted from MON863 and a non-GM corn counterpart, but the staining patterns of the two corn extracts were similar.

In addition to the existing body of evidence indicating that MON863 is as safe as non-GM corn, the results of this study confirmed the predicted lack of binding to Cry3Bb1 by IgE from persons with corn and other food allergies.

2. Methods

2.1. Cloning and expression of Cry3Bb1

Genomic DNA was extracted from MON863 GM corn with a DNeasy Plant Mini Kit (Qiagen, Tokyo, Japan), and the Cry3Bb1 coding sequence introduced into MON863 was amplified by the polymerase chain reaction (PCR). The amplicon was then subcloned into plasmid vector pT7-Blue-T (Merck, Darmstadt, Germany), and the nucleotide sequence was confirmed. An NdeI site and XhoI site were introduced at the 5′ end and at the 3′ end, respectively, of the Cry3Bb1 coding sequence by PCR. The Cry3Bb1 gene fragment was then subcloned into pCold1 (Takara, Ohtsu, Japan) and transformed into pG-Tf2/BL21 (Takara). Expression of the recombinant protein was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). A fusion peptide consisting of 16 amino acid residues including a His tag was added to the N-terminus of the recombinant Cry3Bb1.

2.2. Purification of recombinant Cry3Bb1

After induction of expression of the recombinant protein, 250 ml of the *Escherichia coli* culture was subjected to centrifugation. The *E. coli* pellet was washed in 25% sucrose, 0.1 M Tris–HCl (pH 8.0), and 1 mM EDTA, and it was then resuspended in 3 ml of 10 mM Tris–HCl (pH 8.5) (buffer A) supplemented with protease inhibitors and disrupted by sonication. The suspension was centrifuged, and the supernatant was recovered and applied to a 5-ml HiTrapQ HP column (GE Healthcare, Buckinghamshire, England). The proteins that were adsorbed were eluted in a stepwise manner with 25 ml of buffer A, followed by successive 5 ml volumes of buffer A plus 0.1 M, 0.2 M, 0.3 M, 0.4 M, and 0.5 M NaCl. The fractions eluted with 0.2 M NaCl and 0.3 M NaCl were combined, mixed with imidazole solution to a final imidazole concentration of 20 mM, and then loaded onto a 5-ml HisTrap HP column (GE Healthcare). The proteins adsorbed were eluted in a stepwise manner with a 25 ml volume of 10 mM sodium phosphate (pH 7.4), 0.25 M NaCl (buffer B) plus 20 mM imidazole, and then successive 5 ml volumes of buffer B plus 40 mM, 100 mM, 200 mM, 300 mM, and 500 mM imidazole. The fractions eluted with 200 mM, 300 mM and, 500 mM imidazole were combined, and then concentrated with Centriprep YM-50 (Millipore, Bedford, MA, USA). Imidazole was removed with a 5-ml HiTrap Desalting column (GE Healthcare) and buffer containing 0.1 M Tris–HCl (pH 7.0) and 0.1 M NaCl. During purification each fraction was analyzed by SDS–PAGE and Coomassie brilliant blue (CBB) staining. Polyclonal rabbit antibody Bt Cry3B AP was purchased from Fitzgerald Industries International, Inc. (Concord, MA, USA) and used for the immunoblot analysis.

2.3. Serum samples

Commercially available serum samples from a total of 13 corn allergy patients in the US (PlasmaLab International, Everett, WA, USA) were purchased in 2005 and 2006. Information on the serum samples is shown in Table 1.

In 2003 and later serum samples were obtained from 55 Japanese patients with food allergies who exhibited signs of allergy clinically and were positive for allergen-specific IgE when tested by the Immuno-CAP method (Pharmacia Diagnostic, Uppsala, Sweden). Information on the serum samples is shown in Table 2.

It could not be confirmed if the corn allergy was diagnosed clinically by double-blind, placebo-controlled food challenge (DBPCFC).

Sera from three healthy donors (specific-IgE-negative) in Japan were used as controls. Informed consent had been obtained from all of the food allergy patients and healthy donors. This study was approved by the Institutional Review Board of the National Institute of Health Sciences.

2.4. ELISA

Assay plates (96 wells) were coated with purified Cry3Bb1 (0.2 μg/μL 50 mM sodium carbonate buffer [pH 9.6]/well) at 4 °C overnight. After washing with PBS containing 0.05% Tween 20 (PBS-T), the plates were blocked for 2 h at room temperature (RT) with PBS containing 0.1% casein, and then washed with PBS-T and incubated overnight at 4 °C with serum samples diluted 20-fold in 0.1% casein–PBS. Next, the wells were washed with PBS-T containing 1 M NaCl as described previously (Takagi et al., 2006). The wells were then exposed for 1 h at RT to peroxidase-conjugated goat anti-human IgE antibodies (Nordic Immunology, 1:1000) in 0.1% casein–PBS and washed. Substrate solution (TMB reagent; BD Biosciences, San Diego, CA, USA) was added, and color development was allowed to continue for 5 min. Colorimetric intensity (OD_{450}–OD_{570}) was measured according to the manufacturer's protocol, and the control was measured after adding buffer alone. The control value has already been subtracted from the values shown in Figs. 2 and 3. The experiment was carried out twice, and serum samples were measured in duplicate on each plate. A serum sample was judged to be ELISA-positive, when the absorbance of the sample was greater than the value of the mean plus 5 SD of the control serum values (Yagami et al., 1998).

Coating of the plates with recombinant Cry3Bb1 was confirmed by binding with rabbit antibody Bt Cry3B AP (Fitzgerald Industries International, Inc.) and by failure to bind with normal rabbit serum.

The inter variability and intra-assay variability of the ELISA data were 10.3% and 7.6%, respectively.

Table 1
Serum characteristics. All serum samples were from American patients with corn allergy and were purchased from PlasmaLab International. They were all reactive to multiple food allergens. The anti-Cry3Bb1 IgE ELISA values are after subtraction of the mean buffer control value. The anti-Cry3Bb1 IgE ELISA values of the healthy donors were 0.0173, 0.0231, and 0.0385, respectively.

Serum No.	Anti-Cry3Bb1 IgE ELISA ($OD_{450-570}$)	Immuno-CAP for corn (IU/ml)	Allergens other than corn which bind to IgE
1	0.0295	18.5	
2	0.0350	5.1	
3	0.0260	5.4	
4	0.0225	19.3	
5	0.0560	13.8	
6	0.0270	4.1	
7	0.0135	13.9	
8	0.0200	4.3	
9	0.0270	10.2	Potato, chicken serum proteins
10	0.0090	31.5	
11	0.0090	28.0	
12	0.0175	15.3	
13	0.0135	4.9	Chestnut, walnut