

**Draft *Bacillus thuringiensis*-Corn
Crystalline and
Vegetative Insecticidal Proteins**

Maine Board of Pesticides Control Medical Advisory Committee

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Appendices

- A. Bt Sweet corn labels and registration materials (**Not included here, will be part of the final package**)
- B. Austrian Study and Drs Rice and Wisers' Evaluations
- C. Cry1Ac in vitro Immunological studies
- D. EPA Cancer Classification Schemes
- E. Benchmark dose (BMD) risk assessment methodology

Acronyms

Acronym	Definition
A/G	Albumin/globulin ratio
ADF	Acid Detergent Fiber
ADFI	Average Daily Feed Intake
ADG	Average Daily Gain
ALB	Albumin
ALP	Alkaline Phosphate
ALP U/l	Alkaline phosphatase U/l
ALT	Alanine Aminotransferase
APPT(s)	Activated partial thromboplastin time
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BAS	Basophils
B-cell	Bursa cell
BMD	Bench Mark Dose
BMD ₁₀	Bench Mark Dose
bp	Basepairs
BPC	Board of Pesticides Control
BRAD	Biological Registration Action Document
BSA	Bovine Serum Albumin
Bt	<i>Bacillus thuringiensis</i>
BUN	Blood urea nitrogen
Ca ⁺⁺	Calcium

Acronyms

Acronym	Definition
cCry	Crystalline form of the Bt crystalline protein
CDC	Centers for Disease Control and Prevention
CHOL	Cholesterol
Cl ⁻	Chloride
CP	Crude Protein
CREA	Creatinine
CRW	Corn Root Worm
Cry	Crystalline proteins, Bt
DBI	Double Bond Index
DBIL	Direct bilirubin
DM	Dry Matter
DMI	Dry Matter Intake
DON	Deoxynivalenol
ECB	European Corn Borer
ECM	Energy corrected milk
ELISA	Enzyme-linked immunosorbent assay
EOS	Eosinophils
EPA	Environmental Protection Agency
EPA	US Environmental Protection Agency
FA	Fatty Acids
FAW	Fall Army Worm
FCM	Fat Corrected Milk
FCR	Feed Conversion Ratio g feed/g body weight
FDA	US Food and Drug Administration
FE	Feed Efficiency
FI	Feed Intake
FQPA	Food Quality Protection Act 1996
FQPA SF	Food Quality Protection Act Safety Factor
G	Glufosinate

Acronyms

Acronym	Definition
GGT	Gamma glutamyl transferase
GI	Gastrointestinal tract
GLOB	Globulin
GLUC	Glucose (mg/dl)
GM	Genetically modified
GMO	Genetically Modified Organism
HCl	Hydrochloric acid
HCT	Hematocrit
HGB	Hemoglobin
HMP	High mobility protein
IEL	Intraepithelial lymphocytes
ig	Intragastrin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
ip	Intraperitoneal
K ⁺	Potassium
Kb	Kilo base pairs
LC ₅₀	Median lethal concentration
LD ₅₀	Median lethal dose
LDH	Lactate Dehydrogenase
LDT	Lowest Dose Tested
LL	Liberty Link
LM	Longissimus Muscle
LOAEL	Lowest Observable Adverse Effect Level
LST	Lifetime Study
LUC	Leucocytes
LYM	Lymphocytes

Acronyms

Acronym	Definition
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscle Hemoglobin concentration
MCV	Mean cell volume
ME	Metabolizing Energy
MGS	Multigenerational Study
MIP	Macrophage inflammatory Protein
MON	Monocytes
MUN	Milk Urea Nitrogen
Na ⁺	Sodium
NDF	Neutral Detergent Fiber
NEL	Net Energy for Lactation (Mcal/kg)
NEU	Neutrophil
NGO	Non-Governmental Organization
NMC	n-Methyl carbamtes
NOAEL	No Observable Adverse Effect Level
Non-GM	Non-Genetically modified
NRC	National Research Council
NSPIR	National State Pesticide Information Retrieval System
PCB	Polychlorinated biphenyls
PCR	Polymerase Chain Reaction
PHOS	Phosphorus
PIP	Plant Incorporated Protectant
PLT	Platelets
PMI	Phosphomannose isomerase
PMI	Purina Mills Inc.
PT (s)	Prothrombin time
PTA	Predicted transmitting ability
RABC	Reproductive Assessment by Continuous Breeding
RBC	Red blood cells

Acronyms

Acronym	Definition
RBCW	Red blood cell width
RED	Re-registration Eligibility Decision
RET	Reticulocytes
RR	Roundup Ready
SAP	Scientific Advisory Panel
SCC	Somatic Cell Count
SCM	Solids corrected milk
sCry	Soluble form of the Bt crystalline protein
SDH	Sorbitol Dehydrogenase
SL	Soy lectin
SNF	Solids Non Fat
SPIRS	Silver Platter Pesticide Information Retrieval System
SRBC	Sheep Red Blood Cells
SWCB	South West Corn Borer
TBIL	Total bilirubin
T-cell	Thymus cell
TDN	Total Digestible Nutrients
TMR	Total mixed ration
TP	Total protein
TRIG	Triglycerides
TWA	Time Weighted Average
UMCE	University of Maine Cooperative Extension
VIP	Vegetative Insecticidal Proteins, Bt
WBC	White blood cells

Definitions for Diets

Phrase	Definition
Basal diet	Standard diet, not supplemented with corn
Bt-diet	Variety of corn genetically modified to produce one or more Bt-Cry proteins
Commercial corn diet	Basal diet with commercial corn, origin and growing conditions not reported
Historical controls	Ranges of values for experimental parameters from the lab where the experiment was performed
Isoline	Parental genetic line of corn, without genetic transformation
Literature ranges	Ranges of values for experimental parameters from the available literature
Reference diet	Commercial varieties of corn not related to the Bt or the isoline grown in the same season as the Bt and isoline varieties

Executive Summary

This review of the mammalian toxicology data for Bt-corn and its associated proteins as found in plant incorporated protectants (PIPs) was undertaken by the Board of Pesticides Control (BPC)'s Medical Advisory Committee (MAC). Included in this review are the results from the EPA required studies, toxicity dietary studies found in the literature specific to the Bt-proteins currently being considered for registration in Maine; Cry1Ab, Cry1A.105, Cry2Ab2, Cry3Bb1. In addition, summaries of toxicology and dietary livestock studies for the Bt-proteins of historically relevance in Bt-corn products, Cry1F, Cry34Ab1/Cry35Ab1, and Cry9c are also included. The last of the Bt-proteins reviewed here are Bt-vegetative insecticidal protein (VIP), specifically, VIP3Aa20 found in Syngenta's, MIR 162 corn. The genetic information necessary for production of these proteins have been inserted into 22 PIP products either currently registered or seeking registration by the BPC. The scope of this review also includes an analysis of sweet corn grown in Maine with regard to toxicity of chemical insecticides used. The use of these compounds is expected to decrease if the Bt technology is adopted. Another food safety issue related to the use of Bt-corn is that of a potential reduction in the levels of mycotoxins (secondary metabolites of fungi) in Bt insect protected corn.

EPA's registration process for plant incorporated protectants includes event specific demonstration of the product characterization: genetic transformation process, stability of the inheritance and characterization of the DNA and protein products. Those studies required for the human health assessment include; heat stability and amino acid homology, *in vitro* digestion studies, comparison with known protein toxins and allergens and acute toxicity at high doses in mice. EPA issues Biological Registration Action Document (BRADs) for PIPs and Re-registration Eligibility Decisions (REDs) for chemical pesticides. **Following EPA's review of the aforementioned PIPs, they registered the products and exempted the proteins and the genetic material necessary for production in corn from tolerances.**

The studies reviewed were laboratory studies, *in vivo and in vitro*, using either modified corn or purified proteins. In addition, dietary livestock studies using chickens, swine, cows etc. were summarized. Corn cultivars that are genetically modified to produce Bt proteins are routinely compared to their genetic near isolines and other corn varieties when they are being evaluated in laboratory or field studies. In these equivalency studies levels of protein, fat, ash, moisture, fiber, minerals, fatty acids, amino acids, secondary metabolites and anti-nutrients are compared in the corn. Observed differences were corrected in the formulation of the diets.

The livestock studies may or may not have included diet equivalency portion. In some studies the equivalency data was reported in detail and included pesticide and mycotoxin analysis. In others it was stated that the diets were equivalent. There were occasional statistically significant differences in the parameters in these studies, none of which were deemed biologically significant

In the Cry1Ac *in vitro* studies there were a wide variety of immunological responses observed when the protein was activated by trypsin and high pH prior to exposure. Because these conditions are not observed in the human gastrointestinal tract, the relevancy of these findings is questionable.

With regard to the VIP proteins, the sole toxicity data reviewed were the EPA required studies. Similar to the Cry proteins, there was no acute toxicity observed, the proteins were unstable in gastric fluid and lacked homology to known toxic proteins and allergens.

The chemical pesticide active ingredients likely to be used on sweet corn in Maine are methomyl and three synthetic pyrethroids, esfenvalerate, cyfluthrin and cyhalothrin. The most toxicologically significant reduction in pesticide use on sweet corn is expected to be in Lannate SP (90% methomyl by wt; signal word DANGER, Restricted use based on high human toxicity).

The most significant reduction in chemical pesticide use is expected to be decreases in Lannate uses. If 100% of the sweet corn growers adopted the technology there would be a reduction of between 3,375 lbs and 9,450 methomyl (ai), typical and maximum use rates respectively. The use Bt sweet corn will reduce, but not eliminate the use of chemical insecticides.

It was the consensus of the Medical Advisory Committee that the data required by EPA for registration of Bt corn products for application in sweet corn is inadequate to perform a human health risk assessment. It was also agreed that there is a relative absence of non-industry controlled safety data on Bt foods re: human health—the MAC would support the generation of such data prior to further registrations. (A)

OUTLINE (B)

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SCOPE

BACKGROUND

- EPA's Registration Process for Plant Incorporated Protectants (PIPs)
- Nature and mode of action of Bt-Cry proteins
- Registration status in Maine

Cry Proteins under review for registration in Maine

- Cry1Ab (events: Bt 11, Bt 176, MON 810, KMD1)
- Cry1A.105 and Cry2Ab2 (event MON 89034)
- Cry3Bb1 (events: MON 863 and MON 88017)

Cry Proteins found in Bt sweet and field corn; historical information

- Cry1F (events: TC 625, TC 1507 and DAS-01507)
- Cry34Ab1 and Cry35Ab1 (event: DAS-56122-7)
- Cry9c (Starlink)

Cry1Ac *in vitro* studies with purified soluble Cry1A proteins

Vegetative Insecticidal Proteins

- Differences between VIP and Cry proteins
- VIP3Aa20 in corn (event MIR 162)

Sweet corn grown in Maine

- Chemicals used on sweet corn
- Toxicity of chemicals used on sweet corn

Mycotoxins levels in Bt corn compared to non-Bt-corn

- Studies designed to analyze for mycotoxins residues
- Dietary studies including mycotoxins analysis in the diet composition

SCOPE

This review of the mammalian toxicology data for Bt-corn and its associated proteins was undertaken by the Board of Pesticides Control (BPC)'s Medical Advisory Committee (MAC). The MAC's role is to advise the BPC regarding human health concerns from the use of pesticide products in the state of Maine.

Included in this review are the results from the EPA required studies, toxicity dietary studies found in the literature specific to the Bt-proteins currently being considered for registration in Maine; Cry1Ab, Cry1A.105, Cry2Ab2, Cry3Bb1. In addition, summaries of toxicology and dietary livestock studies for the Bt-proteins of historically relevance in Bt-corn products, Cry1F, Cry34Ab1/Cry35Ab1, and Cry9c are also included. Also, *in vitro* studies performed evaluating the immune responses in mice with the purified protein Cry1A (primarily solubilized Cry1Ac (sCry1Ac) proteins are included. The last of the Bt –proteins reviewed here are Bt-vegetative insecticidal protein (VIP), specifically, VIP3Aa20 found in Syngenta's, MIR 162 corn.

The scope of this review also includes an analysis of sweet corn grown in Maine with regard to toxicity of chemical insecticides used. The use of these compounds is expected to decrease if the Bt technology is adopted. Another food safety issue related to the use of Bt-corn is that of a potential reduction in the levels of mycotoxins (secondary metabolites of fungi) in Bt insect protected corn.

Bt Sweet corn Products under consideration

Syngenta submitted request for registration for the Attribute sweet corn sweet corn with the genetic event, Bt 11 that produces Cry 1Ab protein on October 8, 2008 (EPA# 65268-1) (Syngenta 2008a). The Bt 11 event has been registered at the federal level in field and sweet corn since 1996 and the field corn variety was registered (EPA# 67979-8) in Maine in 2007. The Board of Pesticides Control (BPC) registered Attribute sweet corn in March 2009 (BPC 2009).

In July of 2008, Monsanto (MON) submitted requests to register two new genetic events for use in sweet corn (and field corn) to the Maine Board of Pesticides Control (BPC). One genetic event MON 89034 (EPA# 524-575) is a novel event creating two different Bt proteins Cry1A.105 and Cry2Ab2 for lepidopteron control (EPA 2008a, Monsanto 2008a). The second event results from a classic breeding cross of MON 89034 and MON 88017 (EPA# 524-576). The MON 88017 is currently approved in Maine for use in field corn and controls root worm with the Bt protein Cry3Bb1 (EPA 20008b, Monsanto 2008b).

Recently the Board has received applications for registrations for 3 Syngenta products; MIR 162 (Vip3Aa20) Maize (EPA# 67979-14) (Syngenta 2008b); Agrisure 2100; Bt11 (Cry1Ab) x MIR162 (Vip3Aa20) EPA# 67979-12 (Syngenta 2009a) and Agrisure 3100 Bt11 (Cry1Ab) x MIR162 (Vip3Aa20) MIR604 (mCry3a) EPA# 67979-13(Syngenta 2009b).

Monsanto and Dow AgroSciences have submitted registration request for “Stax” products containing: MON 89034 (Cry1A.105 Cry2Ab2) X MON 88017 (Cry3Bb1) X DAS-59122-7 (Cry34Ab1/ Cry35Ab1). Monsanto’s product has an EPA# of 524-581 (Monsanto 2009) and the Dow AgroScience product an EPA# of 68467-7 (Dow AgroScience 2009).

The labels for the Bt-sweet corn products are found in Appendix A.

Bt-corn Review Status in Maine

The Board of Pesticides Control (BPC)’s Medical Advisory Committee (MAC) reviewed BT 11, Cry1Ab producing, field corn in 1997. The MAC conclusions were:

The Bt 11 corn and its isogenic counterpart are comparable with regard to percent protein, starch and fiber

The MAC report stated that “they did not anticipate a toxicity or allergenicity problem, especially considering that the corn will be used as a feed commodity” (BPC 1997).

Nationally, Bt 11 sweet corn has been in use since 1996. It is currently registered in Vermont and New Hampshire (NSPIRS 2008). Prior to the December 2008, Attribute label change allowing sales to and use by homeowners, the use has been limited to large commercial growers. This makes it likely that this variety of corn is found in processed corn consumed in Maine and in fresh corn coming in from out of state.

BACKGROUND

EPA’s Registration Process

Plant Incorporated Protectants

Bt-proteins found in genetically modified corn are regulated as plant incorporated protectants (PIPs) by the bio-pesticides section of the Environmental Protection Agency (EPA). EPA also registers chemical insecticides (such as synthetic pyrethroids and carbamates). Given the nature of these two types of products, proteins generated in genetically modified food crops and chemical agents with different mechanisms of toxicity,(C) the types of EPA data requirements and risk assessment procedures for these two types of products are quite different.

Regarding the PIPs, EPA requires event specific demonstration of the product characterization: genetic transformation process, stability of the inheritance and characterization of the DNA and protein products. Those studies required for the human health assessment include; heat stability and amino acid homology, *in vitro* digestion studies, comparison (D) with known protein toxins and allergens and acute toxicity at high doses in mice. EPA issues Biological Registration Action

Document (BRADs) for PIPs and Re-registration Eligibility Decisions (REDs) for chemical pesticides.

EPA requires event specific demonstration of the product characterization: genetic transformation process, stability of the inheritance and characterization of the DNA and protein products. Those studies required for the human health assessment include; heat stability and amino acid homology, *in vitro* digestion studies, comparison with known protein toxins and allergens and acute toxicity at high doses in mice. Due to insufficient concentrations of the Bt proteins in the Bt corn, industrial fermentation microbes are transformed with the same method as the corn and the protein products are tested for equivalency. The microbial produced proteins are used in the acute toxicity studies.

Part of EPA's reasoning is that when proteins are acutely toxic, they are toxic in low doses. In addition, there would be no chronic exposure because the proteins break down into their constituent amino acids in the mammalian gut. If a protein is not acutely toxic at doses above the cutoff of 2,000 mg/kg, then EPA assumes that chronic exposure going to occur and does not require mutagenicity, oncogenicity, developmental, sub-chronic or chronic toxicity studies (EPA 2001a). Results from these studies are the basis for the exemptions from tolerances in food and feed (EPA 2001a, EPA 2001b, EPA 2004c, EPA 2008d, EPA 2008e).

EPA classifies all pesticide products according to their acute mammalian toxicity and their ability to induced temporary or permanent skin and eye damage. The scale used is of I to IV, Table 1. All of the Bt-corn products are in Toxicity Category IV, the lowest level of acute hazard from Table 1.

Dietary allergens tend to be resistant to degradation by heat, acid and proteases, to be glycosylated and are present in high concentrations in food. They also have similarities to known allergens and gliadins (EPA 2001a). Gliadins are a heterogeneous mixture of 40 to 60 proteins with molecular weights of approximately 36 kilodaltons. These compounds along with glutenins form gluten (Fennema 1985).

National Academy of Sciences (NAS) under the auspices of the National Research Council (NRC) published a volume titled "Safety of Genetically Engineered Foods" in 2004 (NRC 2004). The NAS document provides a good summary of the types of traditional breeding and genetic modifications used in cultivar creation, including genetic engineering. The genetic engineering methods discussed include: *Agrobacterium* and Biolistic transfers. Interestingly, the most likely source of unintended genetic effects is seen with mutation breeding, chemical mutagenesis and ionizing radiation. These methods are accepted under the National Organic Standards (NRC 2004). The case is made that regardless of the type of breeding, critical analysis of all cultivars both pre-market and post market should be undertaken.

Bernstein *et al.*, 2003 published a mini-monograph on "*Clinical and Laboratory Investigation of Allergy to Genetically Modified Foods*" in Environmental Health Perspectives. They provided a good overview of food allergies, allergens and clinical reactions to exposure to allergenic foods

in susceptible individuals. They identify the biochemical properties of food allergens as: proteins, usually glycoproteins, water soluble, heat and acid resistant, and 10 to 60 kDa molecular weight. They also address the issue of gene origin, known allergenic food or not and the homology with known allergens. These are the criteria EPA uses to evaluate proteins from genetically modified foods and feeds. Some of the issues raised by Bernstein *et al.*, include the lack of an animal model for food allergens, no information on the minimum dose required to illicit a food allergic reaction and the complexity of latex allergies and cross reactivity with various food groups (Bernstein *et al.*, 2003).

Fedoroff and Brown (2004) reiterated the standard tests required by EPA. They also included a quantitative definition of “high levels” in the diets and the degree of homology between the protein of interest, in this case the Bt crystalline proteins and the known allergens. The protein needs to be at a level greater than 1% of the total protein. The allergen protein database includes food allergens, pollen, fungal and insect venoms. The homology with proteins in the known allergen database is tested in sections of protein as small as eight amino acids. There needs to be homology in 80+ amino acids over one third of the stretch of protein for that protein to be suspicious (Fedoroff and Brown 2004).

Chemical Pesticides

For the chemical pesticides, EPA routinely reviews, registers and re-registers these products following risk assessments for the various uses. For chemical pesticides with food uses, EPA requires a full battery of mammalian toxicity tests, including acute (oral, dermal and inhalation), sub-chronic (diet, dermal and inhalation), chronic and cancer bioassays (diet) in multiple species as well as residue studies in plants. These are coupled with exposure assessments, both dietary, and occupational, then incorporated in the risk assessment process. Chemical pesticides are also classified using the toxicity categories found in Table 1.

The nature of the EPA risk assessments and reviews was enhanced with the passage of the Food Quality Protection Act (FQPA) of 1996 in that an additional safety factor (FPQA SF) of 10X was to be used in instances where there was evidence that the developing organisms are more sensitive than the adults, compounds with the same mechanism of action would be evaluated in a cumulative risk assessment and that the standard of care for setting tolerance became “certainty of no harm.” Some or all of these changes in risk evaluation are reflected in the toxicity reviews below.

Nature and the mode of action of Bt Cry-proteins

The mechanism by which delta- or δ -endotoxins affect target organisms was summarized by Gomez *et al.*, 2001, as follows:

“Bt produces insecticidal proteins (Cry toxins) during sporulation as parasporal crystals. These crystals are predominantly composed of one or more proteins, also called d-endotoxins. These toxins are highly specific to their target insect; are safe to humans,

vertebrates, and plants; and are completely biodegradable. The three-dimensional structures of Cry3A and Cry1Aa toxins have been resolved by x-ray diffraction crystallography. The two proteins share many similar features and are composed of three domains. Domain I, extending from the N terminus, a seven-helix bundle, is the pore-forming domain. Domain II consists of three anti-parallel β -sheets, and domain III is a β -sandwich of two anti-parallel β -sheets. Domains II and III are involved in receptor binding, and domain III additionally protects the toxin from further proteolysis (Gomez *et al.*, 2001).

The mode of action of Cry toxins is a multistage process. Crystal toxins ingested by susceptible larvae dissolve in the alkaline environment of the larval midgut, thereby releasing soluble proteins. The inactive pro-toxins are then cleaved at specific sites by midgut proteases, yielding 60–70-kDa protease-resistant active fragments. The active toxin then binds to specific membrane receptors on the apical brush border of the midgut epithelium columnar cells. Therefore, receptors on the brush border membrane are a key factor in determining the specificity of Cry toxins. This specific binding involves two steps, a reversible followed by an irreversible one. After binding, the toxin apparently undergoes a large conformational change leading to its insertion into the cell membrane. The Cry toxin molecules then aggregate through toxin-toxin interactions, leading to the formation of lytic pores, which disrupt midgut ion gradients and the transepithelial potential difference. This disruption is accompanied by an inflow of water that leads to cell swelling and eventual lysis, resulting in paralysis of the midgut and subsequent larval death (Gomez *et al.*, 2001)."

In the native Bt bacteria, the Cry1 pro-toxins are found as bi-pyramidal crystals in the mother cell compartments. Cry2 and Cry3A exist in cuboidal and flat rectangular crystals, respectively. Cry3B exist with an irregular shape. Following consumption by a susceptible insect, the pro-toxins are solubilized in the alkaline midgut and further processed by proteases. The pro-toxins are ~130 kDa in size and the activated toxin is ~65 kDa in size. Digestion by trypsin and chemotrypsin-type enzymes starts at the C-terminus end of the protein (Schnepf *et al.*, 1998). In the mammalian gastrointestinal tract (GI) the proteins are degraded in the acidic stomach (EPA 2001a, EPA 2007d, EPA 2008d, EPA 2008e). In addition mammals lack the specific Cry protein receptors in their GI tracts (McClintock *et al.*, 1995).

The solubility of the Cry-proteins becomes an essential point when evaluating the *in vitro* studies performed with purified proteins. In most cases the purified protein is incubated in pH 9.6 bicarbonate buffer and the soluble protein is released. In a few cases, the purified crystalline version of the protein is used and in at least one case this information is not stated. Vazquez-Padron *et al.*, 1999, demonstrated that the immune reactivity of the soluble protein was much greater than the crystalline version. Details of the individual studies using purified proteins are discussed in the appropriate section below.

The specific Cry-proteins under discussion

The deoxyribonucleic acids (DNA) containing genes in Bt for creating the crystalline proteins exist on a plasmid. Without this plasmid, Bt varieties are indistinguishable from *Bacillus cereus* (Fedoroff and Brown 2004). The δ -endotoxins in the Bt-corn varieties have maintained efficacy against target pests. These proteins are all exempt from tolerance requirements for food and feed (40CFR180.174). The crystalline proteins are:

Cry1Ab is found in corn resulting from the Bt 11 transformation is found in Syngenta field corn products and Monsanto event MON 810

Cry1A.105 is a chimeric protein found in MON 89034 and the cross MON 89034 X MON 88017 the domains from Cry1Ab, Cry1Ac (Domains I and II), Cry1Ac, C-terminus domain and Cry1F (Domain III) make up Cry1A.105

Cry2Ab2 is also found in MON 89034 and the cross MON 89034 X MON 88017

Cry3Bb1 is found in MON 88017 and MON 863

Cry1F is found in events: TC 625, TC 1507 and DAS-01507

Cry34Ab1 and Cry35Ab1 are found in event: DAS-56122-7

Cry Proteins under review for registration in Maine

Cry1ab

EPA Required data

The characterization of the then, Novartis now Syngenta, Bt 11 event and the dietary risk assessment Bt 11 sweet corn is the event summarized by EPA in their 2001 BRAD for Bt Plant Incorporated Protectants (EPA 2001a). Cry1Ab is also produced in Monsanto's MON 810 corn and EPA reviewed this product in the 2001 BRAD (EPA 2001a). In the recently developed MON 89034 event producing Cry1A.105, Cry1Ab constitutes one of the domains.

EPA Product Characterization Cry1Ab

The Bt 11 corn line was developed using the plasmid PZO1502 containing genes for Cry1Ab, *pat* and *amp'*. The *pat* gene results in changes in antibiotic sensitivity used in selection of transformed cultivars *in vitro*. The *amp'* gene was later removed and the guanine: cytosine (GC) ratio was improved for expression in corn. These alterations ended up with production of a truncated version of Cry1Ab production in Bt 11 corn (EPA 2001a).

This truncated version of Cry1Ab, was extracted from the modified corn and compared to that formed in *Escherichia coli* which had also been transformed to produce Cry1Ab. It was verified by biochemical and insect activity assays, that these proteins were similar enough that the *E. coli*

produced protein was used in the mouse feeding study. In addition, inheritance and stability of the proteins was demonstrated in two self crossed generations resulting in 2,320 progeny with the genes intact (EPA 2001a).

MON 810 corn has been modified by ballistic transformation of a Monsanto corn variety with the plasmid construct PV-ZMCT01. Here again the resulting protein is a slightly truncated version of Cry1Ab (EPA 2001a).

EPA Human Health Assessment Cry1Ab

The mouse feeding study with Cry1Ab from transformed *E. coli* was fed to five male and five female mice at dose levels of 3,280 mg/kg protein by gavage. One female did not gain weight between days 7 and 14 days of observation. All animals gained weight by the end of the observation period (EPA 2001a).

Cry1Ab was degraded in gastric fluid *in vitro* within 2 minutes. With regard to heat stability, Cry1Ab protein was inactivated by heat. Cry1Ab is unstable in the presence of pepsin and is not glycosylated. No amino acid sequence homology was detected when Cry1Ab was compared to the database of known allergens or gliadins (EPA 2001a). EPA exempted the Cry1Ab from tolerance (EPA 1995, EPA 1996).

EPA indicated that the fact that the Bt toxins are proteins and that with a few exceptions proteins in the diet present minimal mammalian hazard. They also relied on the history of use for the Bt foliar products used on a wide variety of foods with no recognized adverse effects on humans or other non-target organisms as an indication of low risks (EPA 2001a). Cry1Ab is exempted from the requirement of a tolerance (EPA 1995).

Laboratory studies (in vitro) purified Cry1Ab

Bondzio *et al.*, 2008 investigated the cytotoxicity of Cry1Ab (purified from *E. coli* HB101/pMP) on rumen epithelial cells *in vitro*. The pro-toxin for Cry1Ab used in this study was trypsinized. They used lactate dehydrogenase (LDH) release, WST-1 conversion, ATP content and caspase 3/7 activity to measure Cry1Ab toxicity. Valinomycin and a potassium ionophore were used as positive controls. No effects were observed in the Cry1Ab (concentrations 50 or 100 ng/ml) in either short (24 hr) or long (48 hr) term studies (Bondzio *et al.*, 2008).

Shimada, *et al.*, 2003 exposed cell cultures of bovine hepatocytes to purified Cry1Ab. The Cry1Ab pro-toxin was trypsinized and solubilized, dialyzed against 0.05 M Tris HCl (pH 9.0) overnight. There were no changes in the secretion of albumin or morphology in this assay.

Laboratory studies (in vivo)

Onose *et al.*, 2008 evaluated purified Cry1Ab in a 4week dietary study in Wistar F344 rats with and without gastrointestinal impairment (GI). The study groups of 4 rats per group were; control,

GI control, Cry1Ab (10 ppm) and GI + Cry1Ab (10 ppm). The Cry1Ab in this study was purified and solubilized, put into 10 mM sodium bicarbonate for 5 hrs, prior to use. The GI impairment was induced with famotidine to reduce acidity and indomethacin to cause damage to the small intestine. There were no significant changes in hematology, serum biochemistry or necropsy results (Onose *et al.*, 2008).

Velimirov *et al.* 2008 designed a study to evaluate the reproductive and chronic toxicity of the NK 603, Roundup Ready (RR) X MON 810 (Bt-corn with Cry1Ab) in the OF-11 mice. They used three protocols: multigenerational study (MGS), a reproductive assessment by continual breeding (RACB) and a lifetime feeding study (LTS). The diets used contained either 33% corn from the NK 603 X MON 810 cross or 33% of the isoline (same variety of corn as the Bt line without the genetic modification) of corn. The varieties used in the MGS and the LTS were harvested in 2005. In addition, the Austrian reference corn (A REF) non-GM corn was used as a second control in the MGS. The corn varieties used in the RACB were also grown in Canada and in 2007 (Velimirov *et al.* 2008).

In addition to the standard reproductive endpoints, these investigators looked at gene expression patterns using micro-assay expression profiles (Velimirov *et al.* 2008). There were questions regarding the experimental design and statistical techniques used in this study. At the Board's request, Dr Rice, ME CDC critiqued the reproductive portion of the study and Dr. Wise, USM the micro-assay genetics portion of the study. These critical evaluations are presented in Appendix B.

Immune responses in Balb/c mice receiving MON 810 maize in their diets were investigated by Finamore *et al.*, 2008. The mice used in this study were weanling (21 days) or aged (18 to 19 months). Three diets were used the Bt-corn diet, MON 810 corn, its isoline and a diet made with a variety of commercial corn. Corn constituted 50% of these diets. They were formulated according to the American Institute of Nutrition (AIN)-93G standard. Corn samples were analyzed for mycotoxins; aflatoxins B1, B2, G1, and G2; deoxynivalenol, ochratoxin, zeralenone and found to be below the maximum allowable concentrations. Body weight, food consumption, percentage of lymphocyte cell types, and cytokines were measured (Finamore *et al.* 2008).

No data from the mice fed the commercial diet were reported. There were no differences in body weights or food consumption between the Bt MON 810 and the isoline reported. The percentage of lymphocyte cell types was altered in weanling mice on diet for 30 days in intraepithelial lymphocytes (IELs), spleen and blood. All but B-cells (CD19⁺) had returned to normal after 90 days on study. In the elderly mice differences in the percentage of lymphocyte cell types were observed in IELs and blood. The spleen was negative for this effect (Finamore *et al.*, 2008).

Several cytokines; interleukins: IL-6, IL-13, IL-12p70 and macrophage inflammatory protein (MIP)-1B, increased in weanling mice 30 days on study. With the older mice, weanlings on study for 90 days and elderly mice on study for 90 day an increase in cytokines observed was MIP-1B. The conclusion of the authors was that:

“the significance of these data remains to be clarified to establish whether these alterations reflect significant immune dysfunctions, these results suggest the importance of considering the gut and peripheral immune response to the whole GM crop, as well as age, in the GMO safety evaluation (Finamore *et al.*, 2008).”

Kroghsbo *et al.*, 2008 examined dietary effects of control rice, 60% KMD1 Bt-rice and 60% Bt-rice spiked with 0.1% Cry1Ab on Wistar rats in 28-day (n = 10 females) and 90-day (n = 16 animals per sex per group) feeding studies. The purified Cry1Ab in this study was trypsinized and solubilized, it was dialysed against 10mM sodium carbonate pH 10.5. The resulting doses were 0, 0.6 mg Cry1Ab/kg/day and 70 mg Cry1Ab/kg/day in the 28-day study. The dose levels in the 90-day study were 0 and 0.6 mg Cry1Ab/kg dose levels. The endpoints used were serum concentrations of IgM, IgG and IgA; responses to Sheep Red Blood Cell (SRBC) immunization and mitogen induced cell proliferation.

The results were negative for the Bt rice (not spiked) in both the 28 and 90-day studies. In the 28-day study a positive response was observed for the IgG1 endpoint. The control group demonstrated an IgM response and there was a dose related IgA response that was not statistically significant (Kroghsbo *et al.*, 2008).

Schoeder *et al.*, 2007 examined BMD1 Bt-rice in a 90-day feeding study in Wistar rats. The rice contains Cry1Ab at 15 ppm and the resulting dose levels were 0.54 mg Cry1Ab/kg/day in the rats. There were 16 rats per sex per group, one control and one treated group per sex. The toxic endpoints were behavior, weight gain, hematology, biochemistry, organ weights and histopathology. There were a few statistically significant differences between treatment groups. However, all levels were within the ranges for this breed at this age.

The diets were analyzed for equivalency and also screened for heavy metals, pesticides, and other persistent chemicals as well as bacteria, fungi and mycotoxins. Lead, mercury and cadmium were within limits for these metals in rice. The diets were analyzed for 149 pesticides and were negative at the limit of detection. Polychlorinated biphenyls (PCBs) were not present at a detection limit of 0.0025 ppm (Schoeder *et al.*, 2007).

Livestock studies

Swine

Chowbury *et al.*, 2003 fed Bt 11 corn or it's isoline to pigs, 5 castrated pigs per group, for 28 days and analyzed the gastrointestinal (GI) contents for an intrinsic fragment of DNA found in corn and Cry1Ab fragment of DNA and their respective proteins. The utilized polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) assays to look for DNA and proteins, respectively, in the GI contents. It is noteworthy that there was undigested kernels of corn found in the GI contents. The contents were homogenized before the DNA and protein assays were performed (Chowbury *et al.*, 2003).

As was expected there were fragments of the corn intrinsic DNA markers in the GI content of both Bt 11 and isoline fed pigs. Fragments of the Cry1Ab DNA were found in the Bt 11 corn fed pigs. Neither the corn intrinsic factor or the Cry1Ab DNA fragments were found in blood from the pigs. The GI contents of the Bt 11 corn fed pigs was positive for the Cry1Ab protein determined by ELISA and other immunological tests. It was not feasible to test the blood for the proteins. There were no differences in food consumption, growth rate or food efficiency between the two groups. In addition no differences in general health were observed (Chowbury *et al.*, 2003).

Dairy cows

Phipps *et al.*, 2003, evaluated RR soy meal and MON 810 corn in the diets of lactating dairy cows in terms of equivalency of the feed and the presence of transgenic and endogenous soy and corn DNA in rumen, feces, blood and milk. The diets were equivalent for dry matter (DM), crude protein (CP), starch, acid detergent fiber (ADF), neutral detergent fiber (NDF) and oil. The feed of cows receiving a combination of RR soy meal and MON 810 corn constituted the genetically modified (GM) group (n = 13). The isolines for the RR soy and the MON 810 corn were included in the non-GM diet (n = 5).

The markers used for the GM DNA were 35S promoter gene from the RR soy and Hsp70 Cry1Ab from the MON 810 corn. The markers for the plant endogenous DNA were the soybean lectin (SL) and corn high mobility protein (HMP) genes. To amplify the plant genes, a multi-copy chloroplast gene from *Zea mays* and *Glycine max* (RUBISCO) was included in the study (Phipps *et al.*, 2003).

One difference between the soy and corn components of the diet is the degree of processing. The soy was processed and the result were smaller fragments of DNA (500 base pairs (bp) to 2 Kilo base pairs (Kb) vs the corn (> 23 kb). In the rumen and the duodenal regions of the bovine GI tract feed is in a partially digested state. The liquid portions of the content of these organs as well as the feces, blood and milk tested negative for 35S, Hsp 70 (transgene markers), SL and HMP (plant gene markers) in both GM and non-GM fed cows. The solid phase of the rumen and duodenal contents tested positive for these markers (Phipps *et al.*, 2003).

Analysis for the RUBISCO gene resulted in positive or equivocal results in all samples except for the duodenal liquid phase, 2 GM-fed cows and blood from 1 GM and 2 non-GM fed cows. Further analysis of the RUBISCO data indicated fragments occurring 1176 bp in the ruminal and duodenal digest and 351 bp in the feces (Phipps *et al.*, 2003).

Donkin *et al.*, 2003 investigated the effects of MON 810 corn on lactating cows. The endpoints for these studies include the same dietary equivalency as for poultry. The endpoints include feed intake (FI), feed efficiency (FE), feed conversion rate (FCR), NDF, ADF, dry matter intake (DMI), total digestible nutrients (TDN) and milk components including net energy for lactation in Mcal/kg, (NE_L), fat corrected milk (FCM), solids nonfat (SNF) and somatic cell count (SCC) (Donkin *et al.*, 2003).

One study was identified which evaluated the effects of Bt MON 810 on dairy cows and milk production parameters. Donkin *et al.*, 2003 fed dairy cows MON 810 and its isoline diets containing silage and grain from the respective sources. In a switchback design, cows received their assigned diets for 21 days (three times). The first 14 days were considered an adaptation period and FI and milk quality analysis occurred on days 15 to 21. Two groups of cows were evaluated, the first receiving corn from the 1998 cropping year and the second group from the 1999 cropping year. The protocol for cow care and use was approved by the Purdue University Animal Care and Use Committee (Donkin *et al.*, 2003).

The parameters evaluated in this study were body weight, DMI, FCM, milk production, milk yield (kg feed/kg milk), solids nonfat (SNF), somatic cell counts (SCC), and milk components (fat, protein, lactose). No significant effects were seen for any of these parameters (Donkin *et al.*, 2003).

Poultry

Several varieties of Cry1Ab Bt-corn have been studied in dietary studies in poultry. Aspects evaluated in these studies include diet equivalency in terms of protein, carbohydrates, fats, NDF, ADF, CP, mycotoxin and pesticide levels. Endpoints evaluated include FI, FE, FCR, body weights, organ/tissue weights and the composition of tissues. The poultry studies are performed using broilers (male and female) or laying hens (Taylor *et al.*, 2003a, Taylor *et al.*, 2003b, Brake *et al.*, 2003, Aeschbacher *et al.*, 2005, Rossi *et al.* 2005).

The exposure levels are single dose levels expressed as percent corn in the diet. The diets are referred to as the Bt diets, the isoline diets, commercial and reference diets. Commercial diets are made with corn from commerce and the reference diets are from varieties of corn grown under the same experimental conditions as the Bt and isoline corn. In addition to the comparisons of the Bt-corn and the isoline, and the Bt-corn and the reference varieties, comparisons are also made for literature and national standards for composition of poultry diets (Taylor *et al.*, 2003a, Taylor *et al.*, 2003b, Brake *et al.*, 2003, Aeschbacher *et al.*, 2005, Rossi *et al.* 2005).

The results of the dietary analysis for the poultry studies for Cry1Ab (Bt11, Bt176 and MON 810) are summarized in Table 2. No major differences were observed in the composition of the diets used in these studies. The commercial corn from North Carolina used in the Brake *et al.*, 2003 study had higher levels of crude protein and moisture. This was corrected for in the diet by the addition of sand and ground cardboard (Solka Floc) and poultry fat to equalize the metabolizable energy in the diet. The corn varieties in this study were Liberty Link (LL), glufosinate ammonium tolerant varieties. One group was untreated with the herbicide glufosinate and the other Bt variety treated with the herbicide. Analysis for pesticides, including glufosinate was not performed. Aflatoxin and deoxynivalenol mycotoxin levels were non-detectable with a detection limit in the ppb range. However all four varieties of corn were positive for fumonisin between 8.8 and 20.6 ppm. The fumonisin levels were “not excessive by commercial standards” (Brake *et al.*, 2005).

The only other reported differences between diets was in Aeschbacher *et al.*, was higher CP in one diet. Addition of protease equalized the diet without affecting the overall nutrient value of that one diet. The results of these studies are summarized in Table 3.

Cry1A.105 and Cry2Ab2

EPA Required Data

Two Monsanto products are under consideration for registration in Maine. The first is the genetic event, MON 89034 (EPA# 524-575), a novel event creating two different Bt proteins Cry1A.105 and Cry2Ab2 for Lepidopteron control. The second event results from a classic breeding cross of MON 89034 and MON 88017 creating Cry1A.105 and Cry2Ab2 and Cry3Bb1 (EPA# 524-576) (EPA 2008a, Monsanto 2008a).

Cry1A.105

EPA Product Characterization Cry1A.105

The transformation event used to create MON 89034 utilized the ZMIR245 plasmid with genes for Cry1A.105 (Monsanto 2005a). Cry1A.105 is a chimeric protein composed of portions of Cry1Ab, Cry1Ac, and Cry1F proteins (EPA 2006a). MON 89034 has one copy of the Cry1A.105 expression cassette (EPA 2006a, EPA 2007b, EPA 2007c).

There is significant amino acid homology, structural, biochemical and functional similarities between Cry1A.105 and other CryA proteins (Monsanto 2005a). The overall amino acid homology between Cry1A.105 and Cry1Ac, Cry1Ab and Cry1F is 93.6%, 90% and 76.7% respectively. There are four domains in the Cry1A.105a chimeric protein, three are related to the toxins and one is the C-terminus. Toxin related domains I and II are from the Cry1Ab (same as EPA Human Health Assessment Cry1A.105, Cry1Ac domains I and II) protein and domain III is from the Cry1F. The C-terminus domain is from the Cry1Ac protein (Monsanto 2005b).

Cry1A.105 has a LD₅₀ of greater than 2,072 mg/kg bw in mice. There is no known homology between Cry1A.105 and known toxic proteins and it is readily degraded by heat (EPA 2008d).

EPA concluded that the “potential for Cry1A.105 to be a food allergen is minimal” based on the source of the trait is not a source of allergenic proteins, that there is non homology with known allergens, it is unstable in simulated gastric fluid (30 seconds to degrade) and it is not glycosylated (EPA 2008d). Cry1A.105 is exempt from the requirement of a tolerance (EPA 2006a, EPA 2008d).

Cry2AB2

EPA Product Characterization Cry2Ab2

The transformation event used to create MON 89034 also used the ZMIR245 plasmid with genes for Cry2Ab2 (Monsanto 2005a). MON 89034 has one copy of the Cry2Ab2 expression cassette (EPA 2006a, EPA 2007b).

Cry2Ab2 is present in both Bt modified corn and cotton. In their 2001 exemption from tolerance EPA states that Cry2Ab2 derived from the microbial source (used for acute toxicity testing), and that found in corn and cotton are equivalent with regard to biochemistry and functionality (EPA 2001b). The equivalency of the naturally occurring Cry2Ab2 to the protein purified from the Bt-cotton is established in the EPA BRAD for Cry2Ab2 in Cotton. (EPA 2008f). The 2008 exemption from tolerance for Cry2AB2, EPA covers this protein in all commodities (EPA 2008e).

EPA Human Health Assessment Cry2Ab2

Cry2Ab2 has a LD₅₀ of greater than 1,420 mg/kg bw in mice. There is no known homology between Cry2Ab2 and known toxic proteins and it is readily degraded by heat (EPA 2001b, EPA 2008e). EPA concluded that the “potential for Cry2Ab2 to be a food allergen is minimal” based on the source of the trait is not a source of allergenic proteins, that there is non homology with known allergens, it is unstable in simulated gastric fluid (15 seconds to degrade) and it is not glycosylated (EPA 2001b, EPA 2008e). Cry2Ab2 is exempt from the requirement of a tolerance (EPA 2006a, EPA 2008e).

MON 89034

Dietary Equivalency

MON 89034 expresses both Cry1A.105 and Cry 2Ab2. Drury *et al.*, 2008 evaluated MON 89034 corn for equivalency with conventional corn with regards to protein (amino acid composition), fiber; ADF and NDF, minerals, ash, carbohydrates, fatty acid composition and vitamins. Grain and forage samples were collected from 5 replicated field trials in the US and Argentina (3 replicates per trial composed of the MON 89034 and a conventional control grown during the 2004-2005 growing season). The compositional data were compared to literature values for commercial varieties.

There were statistically significant changes in manganese and vitamin B₂ in grain from Argentina trial; phosphorous (forage from the US trial) and in three of the fatty acids in one or both trials. These differences were within the literature range and the International Life Sciences Institute (ILSI) Crop Composition Database (Drury *et al.*, 2008).

Livestock Studies; Poultry

Similar to the poultry studies described above for Cry1Ab, Taylor *et al.*, 2007 evaluated MON 89034 corn in broiler chickens. This study included diet equivalency in terms of protein, carbohydrates, fats, NDF, ADF, CP, mycotoxin and pesticide levels. Endpoints evaluated include FI, FE, FCR, body weights, organ/tissue weights and the composition of tissues. The poultry studies are performed using broilers (male and female) or laying hens (Taylor *et al.*, 2007)

The exposure levels are single dose levels expressed as percent corn in the diet and controls are the genetic isoline, variety similar to the Bt-corn variety except for the Bt-gene and one or more commercial (com) varieties. In addition to the comparisons of the Bt-corn and the isoline, and the Bt-corn and the commercial varieties, comparisons are also made for literature and national standards for composition of poultry diets (Taylor *et al.*, 2007).

In this study, MON 89034 X NK603 variety expressing Cry1A.105, Cry2Ab2 and the RR genes was compared to its isoline and 6 commercial varieties. There were no differences in the composition of the starter or grower finisher diets and no effects on growth, FI, FE, carcass, organ or tissue weights between the Bt-RR variety and the isoline. There were also no differences in tissue composition in breast or thigh meat (Taylor *et al.*, 2007).

Cry3Bb1

EPA Product Characterization Cry3Bb1

MON 88017 was developed using the Agrobacterium mediated transformation system and plasmid PV-ZMIR39. The marker gene in this variety is *Cp4 epsps* which confers tolerance to glyphosate containing herbicides, RR. These two traits are not inherited independently. The Cry3Bb1 protein in MON 88017 differs from the wild type protein by 7 amino acids. There is enough similarity between the MON 88017 Cry3Bb1 and the MON 863 (currently registered in Maine as Yieldgard Plus (EPA# 524-545) and Yieldgard Rootworm (EPA# 524-528)) Cry3Bb1 that EPA bridged the toxicology studies (EPA 2007d).

EPA Human Health Assessment Cry3Bb1

There are 3 variants of Cry3Bb1 currently used in Bt-modified plants. The exemption from tolerance for Cry3Bb1 lists LD₅₀s of greater than 2,700, 2,980 and 3,780 mg/kg bw in mice for these three variants. (EPA 2004c) There is no known homology between Cry3Bb1 and known toxic proteins and it is readily degraded by heat (EPA 2007d).

EPA concluded that the “potential for Cry3Bb1 to be a food allergen is minimal” based on the source of the trait is not a source of allergenic proteins, that there is non homology with known allergens, it is unstable in simulated gastric fluid (30 seconds to degrade) and it is not glycosylated (EPA 2004c, EPA 2007d). EPA exempted Cry3Bb1 from the requirement for a tolerance in 2004 (EPA 2004c).

Equivalency studies

George *et al.*, 2004 investigated the composition of MON 863 corn vs the isoline, (control); Bt vs reference varieties and Bt vs Monsanto's historical controls. They evaluated proximate components (proteins, fat, ash, carbohydrates and moisture), fiber, amino acids, fatty acids, minerals, vitamins and selected anti-nutrients. The Bt and isoline varieties in a randomized complete block design. The reference corn varieties were 18 varieties were grown in the US and Argentina. Forage and grain samples were analyzed. There were some statistically significant differences between the MON 863 corn and isoline controls. These differences were not seen in both the US and Argentina samples and they were within the ranges for the reference varieties as or the Monsanto historical control corn varieties.

Perschmann *et al.*, 2009 analyzed MON 88017 and its isoline for fatty acid (FA) composition in a variety of plant tissues, leaves and roots. They assessed total FA, saturated FA and the double bond index (DBI). The DBI is equal to the sum of double bonds $\{(1 \times \text{monen}) + (2 \times \text{dien}) + (3 \times \text{trien}) / \text{sum of the \% fatty acids}\}$. The FA profiles in the roots were similar between groups. In the leaves there were higher levels of total FA and a higher DBI in the isoline. This was associated with lower alpha-linolenic acid levels leaves from the Bt leaves. The authors reported a wide variability of the FA profiles in corn and that these variations were within the reference values.

Laboratory Studies; in vivo

In 2006, Hammond *et al.*, published the results of a 90-day dietary rat study using MON 863 corn, its isoline and 6 reference varieties (similar corn hybrids grown in Monsanto test plots) in Sprague Dawley derived rats. The corn was grown in Monsanto test plots. The diets were formulated by Purina TestDiet and conformed with Purina Mills Inc. (PMI) Certified Rodent LabDiet 5002. The grain samples were analyzed for mycotoxins and pesticide residues. There were 20 animals per sex per group. The two treatment levels were Bt corn 11% or 33%. The control was the isoline corn at 11% or 33%. Purina Mills used standard corn to make up the remaining corn component of the 11% groups. The commercial varieties constituted 33% of the diet in the reference corn groups.

Clinical pathology, hematology, serum chemistry, urine chemistry and pathology data were collected and analyzed. This study was statistically evaluated by Monsanto, Hammond *et al.*, 2006 in the original study, reevaluated by Seralini *et al.*, 2007 and further reviewed by an expert panel, Doull *et al.*, 2007. The results are discussed below, Bt vs appropriate isoline control (11% or 33% isoline) and Bt vs reference groups from each of evaluations.

The parameters evaluated by Hammond *et al.*, 2006 included standard body weights, organ weights, blood, serum and urine analysis for all animals. Pathological examinations were done on the 33% Bt group. The statistically significant results as reported by Hammond *et al.*, 2006 for males, Bt vs isoline control diets, are found in Table 4. Those differences for female rats are found in Table 5 Bt vs isoline control diets and Bt vs reference diets, Table 6. While there were

several statistically significant differences, there was only one response with a dose response. The blood glucose levels in female rats increased in a statistically significant manner. However, blood glucose and the other effects observed were within the ranges of the reference groups.

There were no differences in body weights or organ weights, absolute or relative. In the high dose females there was an increase in cumulative body weight gain during week 3 and a decrease in week 4 relative to the reference groups but not the concurrent controls. There were also decreases in food consumption observed in the 11% Bt-corn fed males during week 3 and week 10, but not in the 33% Bt corn fed group.

In the Seralini *et al.*, 2007 re-analysis of these data there were also statistically significant results. Here again there were no statistically significant dose related results. Doull *et al.*, 2007 concurred with the Monsanto study design statistical analysis and conclusions.

Healy *et al.*, 2008 performed a 13 week dietary study in rats using MON 88017 corn. In this study, the same protocol (11% and 33% diets for Bt and its control and 6 reference diets; 20 animals per sex per dose group) was used as above in the Hammond *et al.*, 2006 study. The diets were nutritionally equal and no differences were found in pesticide or mycotoxin levels. The only statistically significant difference in this study occurred in females at the high dose 33% Bt vs the control diets. Here again, this difference was not dose related and within the reference range. There were also no differences in organ weights, absolute or relative or pathology.

Livestock studies

Beef cattle

Vander Pol *et al.*, 2005 performed three studies with beef cattle, one grazing study and two dietary studies. In the grazing study, two groups of animals were used, Bt MON 863 and the isoline control. The feeding studies employed two reference diets in addition to Bt and the isoline. Parameters evaluated in these studies were performance related; body weight, average daily gain (ADG), FE, fat deposition, and yield grade. There were no differences in these parameters in these studies.

Swine

Hyun *et al.*, 2005 evaluated the Bt-corn MON 863 and its isoline in two studies in swine. One study was done in Illinois and the other in Nebraska. The percent of corn in the diet for swine is dependent on their age and body weight. In these studies Bt MON 863, its isoline and two diets formulated with commercial corn were used. The nutrient levels in this study were in accordance with the NRC 1998. The percentage of corn in the diets in relation to age is summarized in Table 7.

Measurements in each study included: body weight, ADG, average daily feed intake (ADFI), FE, carcass and ultrasound measurements. Also, the *Longissimus muscle* (LM) area and chemical

composition was also examined. There were no treatment related differences in these parameters in Study I.

In Study II at the Finisher I growth stage, there were significant changes in ADG (Bt vs two commercial diets) and at the Finisher II growth stage there was a difference in the FE comparing Bt fed swine vs isoline fed swine, but not with the commercial diets. In the second study, when comparing the carcass and ultrasound measurements, there were significant differences in tenth rib backfat thickness between Bt fed swine and those receiving the commercial corn based diets. With regard to the LM measurements, in Study II, the Bt and isoline protein levels in the muscle were higher than in the commercial based diet fed swine (Hyun *et al.*, 2005). Hyun *et al.*, 2005 also concluded that MON 863 was nutritionally equivalent to the control.

Poultry

Poultry studies were done in laying hens to evaluate potential effects on carcass weight, FI and egg laying parameters. The diets were designed to meet the NRC 1994 standards for poultry, with corn constituting 51% of the diets (Scheideler *et al.*, 2008).

The diets, digestive organs, fecal material, blood and the eggs were analyzed for Cry3Bb1. The basal diet was negative for the Bt-protein. As was expected, the Bt diet contained Cry3Bb1. Unexpectedly, the control diet was positive for the protein at a low level (comparable to between 0.5 to 1% wt/wt spiking of the basal diet with the Bt diet). Fecal matter, the crop, small intestines, cecum, and large intestines of laying hens receiving the Bt diet were uniformly positive for CryBb1. In those hens receiving the control diet, no Cry3Bb1 protein was detected in the gastrointestinal tract. There was a substance in the eggs interfering with the analysis for the protein. There were no reported effects on hen body weight, feed consumption or egg production in this study. There was also an interfering substance in blood (Scheideler *et al.*, 2008).

Similar to earlier poultry studies described above (Taylor *et al.*, 2003a, 2003b, 2007) dietary were performed with broilers using Bt MON 810 (Cry1Ab producing corn) X MON 863 (Cry3Bb1 producing corn) (Taylor *et al.*, 2003c) and Bt MON 810 (Cry1Ab producing corn) X MON 88017 (Cry3Bb1 producing corn) (Taylor *et al.*, 2005b). The diets were Bt, isoline and commercial corn diets.

The diets were formulated according to NRC 1994 and checked for mycotoxins and pesticides. The starter diets (55 % corn) were fed on days 1 to 20 and the grower/finisher diets on days 21 to the end of the study, day 42. Equivalency between the diets was established (Taylor *et al.* 2003c; Taylor *et al.*, 2005b).

In the MON 863 study, there were no statistically significant differences between the Bt- group and the isoline group with regard to performance, carcass yield, and tissue (breast and thigh) meat composition. There were some statistically significant differences between the Bt-group and the commercial groups regarding feed conversion, a carcass yield variables. In the MON 863

X MON 810 study no statistically significant differences in any parameters were reported (Taylor *et al.*, 2003c).

In the MON 88017 study, there were no statistically significant differences between the Bt-group and the isoline group with regard to performance, carcass yield, and tissue (breast and thigh) meat composition. There was a statistically significant decrease in adjusted feed conversion between the Bt-group and the commercial groups. In the MON 88017 X MON 810 study, there were also statistically significant differences in adjusted feed conversion between the Bt-group and the commercial groups (Taylor *et al.*, 2005b).

Summary

EPA's registration process for plant incorporated protectants includes event specific demonstration of the product characterization: genetic transformation process, stability of the inheritance and characterization of the DNA and protein products. Those studies required for the human health assessment include; amino acid homology, *in vitro* digestion studies, comparison with known protein toxins and allergens and acute toxicity at high doses in mice. EPA issues Biological Registration Action Document (BRADs) for PIPs and Re-registration Eligibility Decisions (REDs) for chemical pesticides. **Following EPA's review of the aforementioned PIPs, they registered the products and exempted the proteins and the genetic material necessary for production in corn from tolerances.**

The non-EPA required studies reviewed were laboratory studies, *in vivo and in vitro*, using either modified corn or purified proteins. In addition, dietary livestock studies using chickens, swine, cows etc. were summarized. Corn cultivars that are genetically modified to produce Bt proteins are routinely compared to their genetic isolines and other corn varieties when they are being evaluated in laboratory or field studies. In these equivalency studies levels of protein, fat, ash, moisture, fiber, minerals, fatty acids, amino acids, secondary metabolites and anti-nutrients are compared in the corn. Observed differences were corrected in the formulation of the diets. The livestock studies may or may not have included diet equivalency portion. In some studies the equivalency data was reported in detail and included pesticide and mycotoxin analysis. In others it was stated that the diets were equivalent.

There were occasional statistically significant differences in the parameters in these studies, none of which were deemed biologically significant.

CRY PROTEINS FOUND IN BT SWEET AND FIELD CORN; HISTORICAL INFORMATION

Cry1F; (Events: TC 6275, TC 1507 and DAS-01507 in corn)

EPA Required Data

EPA Product Characterization Cry1F

TC 1507 corn was developed by Mycogen to express Cry1F by micro-projectile bombardment. Hybridization patterns indicate that one copy of this gene is present in TC 1507 (EPA 2001c). Cry1F is found in Herculex I (EPA# 29964-3, 68467-2), Herculex XTRA (EPA# 29964-5, 68467-6) (Pioneer Hybrid 2007a, Pioneer Hybrid 2007b, Dow AgroSciences 2007a, Dow AgroSciences 207b) and the Stax products, MON 89034 x MON 88017 x TC 1507 x DAS-59122-7 (EPA# 524-58 1, 68467-7) (Monsanto 2009, Pioneer Hybrid 2009).

EPA Human Health Assessment Cry1F

The exemption from tolerance for Cry1F lists an LD₅₀s of greater than 5,050 mg/kg bw in mice for these three variants. (EPA 2001d) There is no known homology between Cry1F and known toxic proteins and it is readily degraded by heat (EPA 2001d).

EPA concluded that the “potential for Cry1F to be a food allergen is minimal” based on the source of the trait is not a source of allergenic proteins, that there is non homology with known allergens, it is unstable in simulated gastric fluid (5 min to degrade) and it is not glycosylated (EPA 2001c, EPA 2001d). EPA exempted Cry3Bb1 from the requirement for a tolerance in 2001 (EPA 2001d).

Laboratory studies Equivalency

Herman *et al.*, 2004 evaluated the equivalency of TC 6275, Bt-corn producing Cry1F and tolerant to Liberty Link, glufosinate-ammonium herbicide. The corn varieties in this study were Bt, Bt treated with glufosinate-ammonium and the isoline grown at 6 sites in the US and Canada. Forage and grain were analyzed for proximate (protein, fat, ash, moisture), fiber and minerals. The grain was further analyzed for FA, amino acids, secondary metabolites and anti-nutrients.

When comparing the Bt to the isoline the NDF was lower. When comparing the Bt treated with glufosinate-ammonium to the isoline, Ca⁺⁺ and phosphorus were decreased (p < 0.05). In no cases were the consistent changes seen in both Bt varieties and the isoline control (Herman *et al.*, 2004).

Laboratory studies (in vivo)

MacKenzie *et al.*, 2007, evaluated DAS-01507-1, Bt-corn producing Cry1F, in Sprague-Dawley rats in a 90-day study. There were 5 diets used in this study were:

- 11% Bt + 22% commercial corn (Bt 11%)
- 33% Bt corn (Bt 33%)
- 11% isoline + 22% commercial corn (isoline 11%)
- 33% isoline (isoline 33%)
- 33% commercial diet (commercial)

The diets were evaluated for nutrient content and nutritional equivalency. The diets were also analyzed for mycotoxins, anti-nutrients and secondary metabolites. All these parameters were within the published ranges for corn. Verification of the Cry1F protein in the Bt diets was determined using a European Corn Borer (ECB) bioassay. Cry1F activity was observed in the Bt diets, but not the isoline or reference diets (MacKenzie *et al.*, 2007).

Toxicological endpoints assessed in this study were nutritional performance, clinical signs, neurobehavioral, ophthalmological, clinical pathology (hematology, clinical chemistry, urinalysis, pathology (gross and micro), organ and body weights. The statistically significant results in males are presented in Table 10. The statistically significant results in females are presented in Table 11. There were no statistically significant differences in the other parameter measured (MacKenzie *et al.*, 2007).

Laboratory studies (in vivo)

Cry1F and Cry1Ac in cotton seed meal was fed to rats (n = 12 per sex per group) in a 90-day toxicity study. The control groups in this study were cotton seed meal from the isoline and three reference varieties of cotton. The Bt proteins were destroyed in the processing of the cotton to cotton seed meal. They were non-detectable at 0.025 ng/mg (25 ppb). The goal of the study was to determine if there were changes in the cotton seed meal resulting from the genetic modifications which could impact performance, ophthalmological, hematology, clinical chemistry or histopathology in rats (Dryzga *et al.*, 2007).

There were no treatment related effects on mortality, no clinical endpoints, sensory evaluations, body weight, body weight gains or feed consumption reported for animals on study. There were also no statistically significant changes in hematological or blood clotting, organ weights or histopathological parameters (Dryzga *et al.*, 2007).

The differences in ALT and AST between the Bt-group and the isoline were not statistically significant as a result of wide variability. The authors reported statistical outliers as the reason for the variability. The two outliers had underlying liver pathology (Dryzga *et al.*, 2007).

The only statistically significant result reported by Dryzga *et al.*, 2007, was an increase in urine volume in the female Bt-group vs isoline. This was interpreted as a “normal variation” based on the absence of differences in urinary specific gravity and the lack of microscopic lesions in the kidneys. This effect was not observed in the male rats receiving the Bt diet (Dryzga *et al.*, 2007)

Livestock studies

Dairy cows

Faust *et al.*, 2007 investigated TC 1507 corn in dairy cattle. TC 1507 contains the genes for the Bt protein Cry1F and tolerance to Liberty Link herbicide (glufosinate-ammonium). The Bt corn had been treated with the herbicide with 2 sequential treatments.

Animals in this study were lactating dairy cows from two genetic groups; high and average predicted transmittable ability (PTA) for milk fat and protein. The PTA is half an animal's expected breeding value and is equivalent to the genetic worth that is expected to be transmitted to its offspring. They received total mixed rations (TMR) with silage and grain from TC 1507 Cry1F containing corn or its isoline in a crossover study. Two blocks of cows received one of the two diets for 28 days, followed by the alternate diet for 7 days as a transition period. The corn silage and grain samples were evaluated for mycotoxin content and the presence of the Cry1F prior to formulation of the TMR. Deoxynivalenol and fumonisins were present in both samples at concentrations below the FDA action levels. The Bt corn silage and grain were positive for the Cry1F and the isoline corn negative. The diets were formulated to meet the requirements of the NRC 2001 guidelines (Faust *et al.*, 2007).

Performance evaluations included fat composition, proteins, solids lactose SCC and milk urea nitrogen (MUN). Energy calculated milk (ECM) and solids corrected milk values were determined. Blood samples were analyzed for clinical chemistry and hematology parameters (Faust *et al.*, 2007).

There were no statistically significant differences in the chemical profile of the blood samples. With regard to hematology endpoints, there were statistically significant differences between the pretrial, Bt fed group and the isoline profiles. However, the level of mean corpuscular hemoglobin concentrations (MCHC), red cell distribution width (RCDW), plasma proteins and fibrinogen were not significantly different, Bt vs control, the levels for all the groups on study were high than the Iowa state reference ranges (Faust *et al.*, 2007).

Cry34Ab1 and Cry35Ab1 purified protein

Laboratory studies (purified protein in vivo)

Genetic event DAS-59122-7 incorporated in corn results in the production of Cry34Ab1 and Cry35Ab1 proteins. Juberg *et al.*, 2009 investigated acute and 28-day toxicity of Cry34Ab1 and Cry35Ab1 Bt proteins in mice. The source of the proteins was the *Pseudomonas fluorescens* expression system. The proteins were not trypsinized or solubilized prior to use and were shown to be equivalent, biologically and biochemically, to the proteins expressed in DAS-59122-7 modified corn.

The acute study was performed by gavage. The dose levels were purified Cry34Ab1 at 2,700 mg/kg, Cry35Ab1 at 1850 mg/kg and a mixture of Cry34Ab1 and Cry35Ab1 at 482 mg/kg and 1,520 mg/kg, respectively. The vehicle in this study was methylcellulose and there were 5 animals per sex per group. A detailed clinical observation battery was done pre-dosing, and on test days 2, 8 and 15. Pathological examination occurred at the end of the study Juberg *et al.*,

2009. There were no deaths in any groups in this study. In addition, there were no clinical or pathological effects reported (Juberg *et al.*, 2009).

In the 28-day dietary study the dose groups (n = 5 per sex per group) were control; Purina Mills Institute (PMI) 5002, bovine serum albumin (BSA) spiked PMI 5002, PMI 5002 amended with purified Cry34Ab1 and cry35Ab1. The makeup of the diets, nominal concentrations and doses calculated based on dietary intake, is outlined in Table 12. The diets will be referred to as: control, BSA, and low medium or high Bt. In-life observations included morbidity, mortality and detailed clinical observations. Gross necropsy and histopathology were performed following sacrifice (Juberg *et al.*, 2009).

One female in the control group died on day 7 of the study. The only clinical observation in study was focal dermatitis in one high dose male. No differences were seen in body weights or gross pathology. Feed consumption was statistically significantly higher in the all groups of males on days 2 and 3 compared to BSA, but not the untreated controls. This effect was transient and not observed in females (Juberg *et al.*, 2009).

Examination of the eyes indicated a well formed nucleus in one male in the eye of one male in the intermediate group pre-exposure resolved by day 27. There were well defined nuclei in both eyes of a low dose female which developed by day 27. No similar lesions were observed at the higher dose levels. There was an increase in neutrophils in the high dose males which was attributed to an abscess in one male. The eosinophils were higher in the BSA group than the untreated control and the treatment groups. There was a statistically significant increase in serum chloride in both males and females in the low dose group only (Juberg *et al.*, 2009).

Cry1F, Cry34Ab1 and Cry35Ab1 (Cross of Events DAS 01507-1 X DAS 59122-7)

Appenzeller *et al.*, 2009 investigated the variety of corn resulting from the cross DAS 01507-1 X DAS 59122-7 in a 90 day feeding study in rats. This variety of corn expresses the Bt proteins Cry1F, Cry34Ab1 and Cry35Ab1. The test groups were fed diets containing the Bt variety, its isoline and 3 varieties of reference corn at 34% of the PMI 5002 standard diet. The biological activity of the Cry1F protein was determined with a European Corn Borer (ECB) bio-assay. The amount of Cry34Ab1 and Cry35Ab1 proteins is not high enough in the grain to be active against corn root worm (CRW). The proteins in the Bt diet were confirmed using enzyme-linked immunosorbent assay (ELISA) methods. The corn was analyzed for equivalency, mycotoxins and pesticides (Appenzeller *et al.*, 2009).

The diets were found to be nutritionally equivalent. Mycotoxins and pesticides, if present, were below action levels or tolerances, respectively. The presence of the Bt-proteins was confirmed in the test diet and their absence demonstrated in the isoline and reference corn (Appenzeller *et al.*, 2009).

also no changes in urinalysis, body or organ weights and histopathology observed in this study. There were increases in RBC and HCT and a decrease in MCH in the female rats, Bt vs isoline.

This was not considered compound related because the differences were 4 to 5 % and within the ranges for the reference groups and historical controls. The clinical chemistry effects observed at a statistically significant level were also observed in females. These were an increase in sodium and a decrease in chloride. These results were also within the range for reference groups (Appenzeller *et al.*, 2009). In the Juberg *et al.*, 2009 study (discussed above) in mice receiving Cry34Ab1 and Cry35Ab1 proteins in their diets there was an increase in chloride, but only at the lowest dose tested (LDT).

Cry9C (Starlink)

The registration of Starlink, Bt-modified corn, was approved by EPA, for non-human food uses in 1998. Human food uses were not allowed because of concerns about allergenicity due to the fact that this protein was not easily degraded in simulated gastric fluid. Starlink was sold by Aventis Corporation in 1998, 1999 and 2000. Non-governmental groups (NGOs) began finding Cry9C and the DNA needed to produce it in yellow corn meal products in 2000. Cry9C is found in naturally occurring Bt subspecies (spp) *tolworthi* and is not found in commercial Bt insecticides (SPIRS 2007) (EPA 2008j).

The biochemical characteristics of Cry9C from Starlink corn used by EPA in its decision to register it only in field corn were: it is a 68.7 kDa toxin, stable to trypsin and pepsin digestion pH 2.0, stable in simulated gastric fluid (4 hrs), and stable to heat (10 min at 90C) (Bucchini and Goldman 2002). The EPA Scientific Advisory Panel (SAP) in addition to the biochemical data above reviewed data generated after the Starlink event. These studies included the rationale that the Cry9C protein may be glycosylated in plants, in the Brown Norway rat, intact protein could be retrieved from the blood and exposure caused allergic responses. No clinical observations or ophthalmological effects were reported during the study (Bucchini and Goldman 2002).

Given these data, the SAP met in November 2000 and concluded that “Cry9C protein has a medium probability of being a potential allergen” based on a finding of multiple characteristics associated with known allergens. The SAP also concluded that there was a “low probability to sensitize some individuals” to the protein based on the level of Cry9C in corn, the amount of Starlink corn expected to have entered the human food supply and that Starlink would be co-mingled with other varieties of yellow corn during processing (EPA 2008j).

Teshima *et al.*, 2002, fed female rats and mice diets with up to 50% heat treated corn meal (with Cry9C) for 13 wks. They evaluated these animals for immune responses and saw no changes in immune parameters (Teshima *et al.*, 2002).

At the request of the Food and Drug Administration (FDA) the Center for Disease Control and Prevention (CDC) performed an epidemiology study on individuals who had reported allergic reactions following ingestion of corn products known to contain Cry9C from Starlink corn. The CDC found that “these findings do not provide any evidence that the reactions that affected people experienced were associated with hypersensitivity to the Cry9C protein” they also pointed out the importance of evaluating the allergic potential of genetically modified foods before they

are available for human consumption (CDC 2001).” It is of interest that EPA changed their Bt-events in corn registration policy; they will no longer issue a registration for only field corn.

Cry Proteins found in cotton

Cry1Ac

Since the late 1990s Cry1Ac has been evaluated as an adjuvant for vaccines. An adjuvant is needed in the immunization process, if the pathogen in question lacks the ability to illicit an immune response. These responses of the immune system following interperitoneal (ip), intragastric (ig), intranasal (nasal) or rectal may be serum and/or mucosal responses. The antibodies measured include IgG, IgM and IgA.

As a reminder, the Cry pro-toxins are processed by trypsin-like and chymotrypsin-like proteases (trypsozined) and solubilized in the alkaline midgut of susceptible insects prior to the binding to the receptors. The molecular weights of the pro-toxins decrease from ~ 130 kDa to 65 kDa following trypsonization (Schnepf *et al.*, 1998). The Cry1Ac proteins used in the studies below, unless otherwise specified are purified and solubilized, but not trypsozined.

Vazquez-Padron *et al.*, 1999 evaluated the ability of purified soluble pro-toxin Cry1Ac (sCry1Ac) versus the crystalline form of the Cry1Ac (cCry1Ac) pro-toxin from transformed *E. coli* to illicit systemic and/or mucosal responses in female Balb/c mice. Following ip administration of sCry1Ac and cCry1Ac there were positive serum responses, increases in IgM and IgG for both forms of the protein. The response to cCry1Ac was 10X lower for the IgM response and 2X lower for the IgG response. The IgA response was positive for sCry1Ac and negative for cCry1Ac administered ip (Vazquez-Padron *et al.*, 1999).

Following ig administration of sCry1Ac and cCry1Ac there were positive serum responses, increases in IgM and IgG for both forms of the protein. The response to cCry1Ac was 10X lower for the IgM response and 5X higher for the IgG response. The IgA response was positive for both sCry1Ac for cCry1Ac administered ip (Vazquez-Padron *et al.*, 1999).

The mucosal responses to ip administration of sCry1Ac and cCry1Ac were negative for IgM, and for IgG and IgA. The positive responses were of the same magnitude. Regarding ig administration of sCry1Ac and cCry1Ac, the responses were positive for sCry1Ac for IgG and IgA and negative for IgM. The response for cCry1Ac were uniformly negative (Vazquez-Padron *et al.*, 1999).

The author’s explanation for the differences between these two forms of the proteins is due to the different biophysical properties. From an immune response perspective:

“To process the protein crystals, antigen presenting cells (APC) require an initial solubilization step for partial proteolysis to occur in the class II MHC system. When

cCry1Ac is administered via ip, pro-toxin crystals are mostly taken up by macrophages and possibly other APC cells, which process the antigens in and acidic lysosomal vesicles. Solubilization of the cCry1Ac normally occurs at high pH values, a fact that may hinder the proteolytic processing of crystals necessary for releasing immunogenic peptides (Vazquez-Padron *et al.*, 1999).”

Vazquez *et al.*, 1999, Moreno-Fierros *et al.*, 2000, Moreno-Fierros *et al.*, 2002, Esquivel-Perez and Moreno-Fierros 2005, performed a series of immunological studies with sCry1Ac alone, sCry1Ac: with Hepatitis B surface Antigen (HBsA), with BSA, with *Streptococcus pneumonia* capsular polysaccharides (CPS6B), with Pneumovax P23 and with HIV-1 C4/V3 peptide T1SP10MN(A) and with the modified HIV-1 C4/V3 peptide, mT1SP10MN(A) in mice. These studies indicate that the soluble non-trypsinized Cry1Ac has profound effects on the immune system. In addition, Guerrero *et al.*, 2004 compared the immune activity of Cry1A protoxins and toxins in the same mouse model. The specific results of these studies are summarized in Appendix C.

Summary

In the Cry1Ac *in vitro* studies there were a wide variety of immunological responses observed when the protein was activated by trypsin and high pH prior to exposure. Because these conditions are not observed in the human gastrointestinal tract, the relevancy of these findings is questionable.

Vegetative Insecticidal Proteins

In addition to the Cry proteins, native strains of Bt have vegetative insecticidal proteins (VIP) which are also active as insecticides. These proteins differ from the Cry proteins in that they are formed in the vegetative stage of the bacteria lifecycle (Ramasamy *et al.*, 2007) and act on a different receptor in the insect gut decreasing the likelihood of insect resistance (Fang *et al.*, 2007, BPC 2007, EPA 2007 i). The VIP proteins include VIP1 and VIP2 proteins with activity on corn rootworm. These proteins are not currently being considered for registration. VIP3A proteins are active against Lepidoptera (Fang *et al.*, 2007).

VIP3Aa20

EPA Required Data

The VIP3A, protein VIP3Aa20 in corn and its close relatives, native VIP3Aa1 and VIPAa19 (engineered into cotton) are under consideration in this review. VIP3Aa19 and VIPAa20 differ from the native protein in two amino acids (EPA 2007h, EPA 2007i). EPA determined that these proteins, VIP3Aa19 and VIP3Aa20 along with VIP3A from transformed *E. coli* are equivalent and bridged the data from VIP3A and VIP3Aa19 to support the registration of VIP3Aa20 (EPA 2007i).

The event MIR 162, the genes for VIPAa20 and the marker phosphomannase isomerase (PMI) were inserted into corn using the pNOV1300 vector (EPA 2007h, EPA 2007i). The resulting VIP was VIP3Aa20, a protein which is 89 kDa in size and shares > 99.7 % homology with its native counterpart VIP3Aa1 (Syngenta 2007c).

The acute LD₅₀s in mice were determined at > 3,675 mg VIP3Aa19 protein /kg and > 5,000 mg VIP3A protein /kg. EPA used the VIP3Aa19 and VIP3A LD₅₀s for VIP3Aa20 in their human health and safety review as well as the exemption from tolerance (EPA 2007h, EPA 2007i, EPA 2007j, EPA 2007k, EPA 2008l). There were no clinical signs reported in the LD₅₀ studies.

In their evaluations of the potential for allergenicity of these products, EPA concluded that the VIP3A proteins were not glycosylated, have no resemblance to known allergens and are rapidly degraded in simulated gastric fluid (EPA 2007i, EPA 2008l). The VIP3Aa20 concentration in corn kernels is between 24.6 to 40.3 ug VIP3Aa20 protein/ g dry weight (EPA 2007i).

Summary

With regard to the VIP proteins, the sole toxicity data reviewed were the EPA required studies. Similar to the Cry proteins, there was no acute toxicity observed, the proteins were unstable in gastric fluid and lacked homology to known toxic proteins and allergens

SWEET CORN GROWN IN MAINE

Acreage

The National Agricultural Statistics Survey (NASS) estimates the number of acres of sweet and field corn grown in Maine on an annual basis. Annual acreage between 2003 and 2007 for sweet corn ranged from 2,100 acres in 2006 and 2,300 acres in 2004 (BPC 2008).

Another way to estimate the acreage of sweet corn is based on the sales (and presumable use) of Lannate SP. Lannate SP is a 90% methomyl water-soluble powder in water-soluble packets used on sweet corn at 8 oz/A (0.5 lbs product/A; 0.45 lbs ai/A) (DuPont 2008a). Using this method, between 1,054 and 1,523 acres were treated with Lannate. The other assumption made was that half the acreage was treated with Lannate and the other half with one of several synthetic pyrethroids. This results in total acreage of between 2,108 and 3,046 acres of sweet corn in Maine.

The number of organic sweet corn acres, here again rounded to the closest 100th is about 100 acres per year. These farms are primarily located in the lower 1/3 of the state hugging the coast (Sideman 2008). The best guess from the UMCE sweet corn specialist is that the certified acreage would be less than 5% of the total produced in Maine (Handley 2008). Using the UMCE estimates total sweet corn grown in Maine is ~ 3,500 acres and < 5% of that is organic (175 acres) (Handley 2008).

Chemical Treatment of Sweet Corn

Lannate SP (90% methomyl by wt) is used on at least 50% of the sweet corn raised in Maine. The approximate number of sprays for a given planting may range from 2 to 5. This means that a single planting may receive anywhere from 1 to 8 sprays, depending on the season, level of pressure, and assuming that the grower is monitoring the pest populations (Handley 2008). Growers not using IPM will probably follow a 5-day schedule (Titus 2008, UMCE 2008).

The products likely to be used (Titus 2008, UMCE 2008) on conventional sweet corn are summarized in Table 13.

Chemical Treatments

Cyfluthrin, cyhalothrin and esfenvalerate (and their respective isomers) are type 2 synthetic pyrethroid insecticides. These compounds contain a cyano group on the α carbon of the alcohol moiety. Exposure to these compounds in rats results in the CS syndrome; pawing and burrowing behavior, profuse salivation, movement disorders: coarse tremors, choreoatetosis and clonic seizures (Klaassen 2008).

Cyfluthrin, cyhalothrin and esfenvalerate are synthetic pyrethroids. The chemistry of the synthetic pyrethroids is quite complex. There are multiple chiral centers and multiple isomers denoted with Greek letters. As a rule, the biological activity both as an insecticide and as a mammalian toxicant resides in specific sets of isomers. In the days when these products were first registered, the technology for isolating the isomers was not available. For example, cyfluthrin was first registered in 1989, and currently there are approximately 145 active products registered for use on a wide variety of sites including agricultural, indoor/outdoor domestic dwellings, ant dens/mounds, and stored product pests. β -cyfluthrin was first registered in 1995 and currently there are approximately 27 active registrations (EPA 2007a)

Cyfluthrin and β -Cyfluthrin

Cyfluthrin consist of four diastereoisomeric [any enantiomers that are not mirror images in a group of optical isomers in compounds with asymmetric carbon atoms (Morris 1992)] pairs of enantiomers [a pair of chemicals with the same chemical composition that are mirror images that are not identical, optical isomers with chiral centers (Morris 1992)]. The isomeric composition of cyfluthrin and β -cyfluthrin is found in Table 14.

EPA's 2002 evaluation of the relationship between cyfluthrin and β -cyfluthrin

“EPA has evaluated the available toxicity data and considered their validity, completeness, and reliability as well as the relationship of the results of the studies to human risk. EPA has also considered available information concerning the variability of the sensitivities of major identifiable subgroups of consumers, including infants and children. The nature of the toxic effects caused by cyfluthrin and its enriched isomer,

beta-cyfluthrin are discussed in Table 1 of this unit as well as the no-observed-adverse-effect-level (NOAEL) and the lowest- observed-adverse-effect-level (LOAEL) from the toxicity studies reviewed. Beta-cyfluthrin is an enriched isomer of cyfluthrin. Bridging data on beta-cyfluthrin were submitted so that the toxicity of beta- cyfluthrin could be compared with that of cyfluthrin and the data bases could be combined to form one complete data base for both chemicals (EPA 2002d).”

“Beta-cyfluthrin is an enriched isomer of cyfluthrin. Currently, residues of beta-cyfluthrin are covered by the tolerances for cyfluthrin. In accordance with EPA preferences regarding tolerances for synthetic pyrethroids and their isomers, Bayer CropScience has proposed establishment of separate tolerances for beta-cyfluthrin. The proposed beta-cyfluthrin tolerances are at the same levels as those for cyfluthrin (EPA 2008h).”

Cyhalothrin, γ -cyhalothrin, and λ -cyhalothrin

γ -cyhalothrin (CAS# 91465-8-6), and λ -cyhalothrin (CAS# 76703-62-3) are contained within the chemical cyhalothrin (CAS# 68085-85-8). Cyhalothrin consists of four isomers, λ -cyhalothrin consists of two of these isomers and γ –cyhalothrin is the single active isomer contained in both. The chronic studies were conducted on cyhalothrin (EPA 2004a).

EPA’s 2002 evaluation of cyhalothrin (prior to purification of the γ -isomer)

“Cyhalothrin and lambda-cyhalothrin are basically the same chemical, the differences are found in their stereo chemistry and the number of isomers in each mixture. Cyhalothrin consists of four stereo-isomers in each mixture. Cyhalothrin consists of four steno isomers while lambda-cyhalothrin is a mixture of the two isomers. The two lambda-cyhalothrin isomers are contained in cyhalothrin and they represent 40% of the cyhalothrin mixture. The major studies submitted to the Agency were conducted with cyhalothrin. However, these studies are used in support of registration for both mixtures. There is evidence, based on sub-chronic studies in rats, that the two mixtures are not biologically different with respect to their mammalian toxicity (EPA 2002c)”.

EPA’s 2008 evaluation of relationship between the cyhalothrin mixtures

“Gamma-cyhalothrin is a single, resolved isomer of the pyrethroid insecticide cyhalothrin. As such, it shares physical, chemical and biological properties with both cyhalothrin and lambda-cyhalothrin, which are mixtures of 4 and 2 isomers, respectively. Gamma-cyhalothrin is the most insecticidally active isomer of cyhalothrin/lambda-cyhalothrin, and thus the gamma-cyhalothrin technical product is considered a refined form of cyhalothrin/lambda-cyhalothrin that has been purified by removal of less-active and inactive isomers. Therefore, similar levels of insecticidal efficacy for gamma-cyhalothrin can be obtained with significantly reduced application rates as compared with either cyhalothrin or lambda-cyhalothrin. EPA has previously concluded that residue data supporting registered uses of lambda-cyhalothrin are sufficient to support registration of

gamma- cyhalothrin for the same uses, as long as the use rates of gamma- cyhalothrin are no greater than half the corresponding use rates of lambda-cyhalothrin. The proposed application rates of gamma-cyhalothrin for the requested new uses (considered herein) are no greater than half of the corresponding, existing application rates for similar registered uses of lambda-cyhalothrin (EPA 2008g).”

“Tolerances are currently established under 40 CFR 180.438 for residues of lambda-cyhalothrin in food-handling establishments. Through the use of bridging data, the toxicology database for gamma-cyhalothrin is complete using developmental, reproduction, chronic (rodent), and oncogenicity studies conducted with cyhalothrin and lambda-cyhalothrin. The nature of the toxic effects caused by lambda-cyhalothrin as well as gamma-cyhalothrin are discussed in detail in the Federal Register of September 27, 2002 (67 FR 60902)(FRL-7200-1). Therefore the toxicology database for gamma-cyhalothrin when bridged with cyhalothrin and lambda-cyhalothrin are complete for purposes of supporting the proposed use in food handling establishments (EPA 2008g).”

Fenvalerate and Esfenvalerate

Fenvalerate is a racemic mixture of four pyrethroid isomers (S,S; R,S; S,R; and R,R). The CAS# for fenvalerate, stereochemistry unstated is 51630-58-1 (Pesticide Manual 2003). Technical Asana (esfenvalerate) is enriched in the insecticidally active S,S-isomer (84%). Esfenvalerate is S-(R*,R*)-4-Chloro-alpha-(1-methylethyl)benzeneacetic acid, cyano(3-phenoxyphenyl)methyl ester (CAS#. 66230-4-4 and CAS# 66323-4-4).

In 1998 EPA issued a set of tolerances and reviewed toxicity studies for either fenvalerate or esfenvalerate (EPA 1998a). The pesticide registrations for fenvalerate were voluntarily canceled in 2003 (EPA 2008i).

The synthetic pyrethroids have not undergone a full post FQPA of 1996 re-assessment. In other words, EPA has not yet published the RED for these three compounds. They are in the re-registration queue. However, since 1996 food tolerances have been issued for all of these compounds and the toxicity reviews below are primarily taken from these documents.

Methomyl, the carbamate in the group of insecticides used on sweet corn in Maine has gone through not only the FQPA level review, an RED (EPA 1998b) has been published and it is included in the 2007 cumulative risk assessment for n-methyl carbamates (NMC) (EPA 2007e). The review of methomyl reflects this level of review.

Chemical Insecticide Toxicology Summary

The toxicity endpoints for the toxicity of the chemical pesticides used in sweet corn culture in Maine is presented in Table 14.

Cyfluthrin

Cyfluthrin and β -cyfluthrin are synthetic pyrethroid insecticides registered for use on sweet corn under the brand names Baythroid 2E (EPA# 264-745) and Baythroid XL (264-840), respectively (Table 13). The risk assessment for cyfluthrin was done in 2002 (EPA 2002d), subsequent tolerance actions, EPA 2008h, refer back to this risk analysis.

Sub-chronic Cyfluthrin Toxicity Studies

Sub-chronic (28 day to 90 day) feeding studies have been done with either cyfluthrin or β -cyfluthrin in rats and dogs. The NOAELs from these studies ranged from 2 mg/kg/day in the 28-day dog study with β -cyfluthrin to 28 mg/kg/day in female rats fed cyfluthrin for 90 days. The effects observed at the LOAELs included decreases in body weight gain, food consumption, food efficiency, nervous system effects: gait abnormalities, increases in salivation and nervousness, vomiting and (dogs) and changes in hematology, and decrease in organ weights. Necrosis around the neck and head were also observed in the 90-day rat study using the β -cyfluthrin (EPA 2002d).

Twenty-one/ twenty-eight day dermal studies were done in the rat, using cyfluthrin. The dermal NOAEL was 113 mg/kg/day and the systemic NOAEL was 376 mg/kg/day. Effects observed at the dermal LOAEL were gross and histological skin lesions. Systemic effects at the LOAEL were decrease food consumption and red nasal discharge and urine staining (EPA 2002d).

Several inhalation studies were reported for cyfluthrin using the rat model. The lowest NOAEL, 0.02 mg/kg/day was from the 90-day rat study. The effects observed at the LOAELs included decreases in body weight gain, food consumption, food efficiency, clinical signs of neurotoxicity in the rats, increase in mortality, clinical signs of toxicity, hypothermia and changes in hematology, increase in urinary pH and piloerection with un-preened coats (EPA 2002d).

Cyfluthrin Developmental Studies

Both oral developmental studies were performed using β -cyfluthrin and cyfluthrin in rats and rabbits. The maternal NOAELs were 3 mg/kg/day in the β -cyfluthrin study and greater than 10 mg/kg/day in the cyfluthrin study. The developmental NOAELs were 10 mg/kg/day in the rat β -cyfluthrin study and greater than 10 mg/kg/day (highest dose tested, HDT) in the cyfluthrin study. Effects in the dams at the LOAEL were decrease in body weight and decrease in food consumption in the β -cyfluthrin rat study. Developmental effects in the β -cyfluthrin rat study were a decrease in pup body weight and an increase in skeletal variations. The LOAEL was determined for in the developmental study in rats using cyfluthrin (EPA 2002d).

The maternal NOAEL in the oral rabbit developmental study using cyfluthrin was 20 mg/kg/day. Effects at the LOAEL (60 mg/kg/day) were decrease in body weight gain and feed consumption in the dams during the dosing period. The development NOAEL was 180 mg/kg/day, HDT, therefore the LOAEL was not determined (EPA 2002d).

Two pre-natal and one post-natal inhalation studies were done with cyfluthrin in rats. The NOAEL was not determined from one of the prenatal studies, the LOAEL was the lowest dose tested (LDT) < 0.125 mg/kg/day (calculated dose). Maternal effects seen at this dose included a decrease in body weight gain and food efficiency. The developmental NOAEL was 0.125 mg/kg/day and the LOAEL was 0.692 mg/kg/day. Developmental effects at the LOAEL were decreased pup body and placental weights and reduced ossification (EPA 2002d).

In the second pre-natal inhalation study (combination of two inhalation studies) the NOAEL was 0.299 mg/kg/day. The LOAEL was 1.277 mg/kg/day. Maternal effects at the LOAEL were increases dyspnea, piloerection, reduced motility un-groomed coats and eye irritation. The developmental NOAEL from this study was 0.16 mg/kg/day. Developmental effects seen at the LOAEL of 0.299 mg/kg/day were an increase in the number of runts and skeletal abnormalities (EPA 2002d).

There was also a post-natal rat inhalation developmental study summarized by EPA. In this study the maternal NOAEL was 24 mg/kg/day (HDT) and the NOAEL in the offspring was 2.48 mg/kg/day. Offspring effects at the LOAEL were clinical toxicity, and an increase in spontaneous motor activity in females at 4 months after exposure (EPA 2002d).

Cyfluthrin Reproductive Studies

There were four cyfluthrin multigenerational rat studies summarized by EPA in their 2002 tolerance final notice. In the EPA acceptable guideline study the parental NOAELs were 3 mg/kg/day for males and 4 mg/kg/day for females. The offspring NOAEL was 9 mg/kg/day. The LOAELs were 9 mg/kg/day for males and 10 mg/kg/day for females for the parental generation. Effects at these doses were reduction in body weight and food consumption. The offspring LOAELs in this study was 19 mg/kg/day. At this dose, there were coarse tremors during the lactation period and a decrease in mean litter weight (EPA 2002d).

The other three reproduction studies were supplemental and NOAELs were higher than that in the guideline study described above. Effects at the LOAELs in these studies included those seen above and hind leg splay and ataxia at 59.6 mg/kg/day in one study (EPA 2002d).

Cyfluthrin Chronic-Oncogenicity Studies

The three chronic feeding studies performed in dogs, resulted in an overall NOAEL of 2.43 mg/kg/day in males and 3.61 mg/kg/day in females. Effects at the LOAELs were clinical signs, gait and postural abnormalities as well as vomiting and diarrhea.

The combined oncogenicity/chronic studies in rats and mice were negative for oncogenic effects. The NOAELs for the studies done with the technical grade active ingredient (93.3% or 94.7%) were 31.9 mg/kg/day in male mice and 140.6 mg/kg/day in female mice. The NOAELs in the rat were 2.6 mg/kg/day in males and 3.3 mg/kg/day in the females. Effects at the LOAELs were and decrease in body weight and body weight gain, food consumption, clinical signs and changes in

pathology in the mice and decrease in body weight gain seen in rats (EPA 2002d).

The current EPA cancer classification for cyfluthrin is “not likely to be a human carcinogen” (EPA 2006b). EPA’s cancer classification criteria are found in Appendix D.

Cyfluthrin Neurotoxicity Studies

Three hen neurotoxicity studies were negative for delayed neurotoxicity and effects on neurotoxic esterase (NTE). Rats received β -cyfluthrin by gavage in an acute neurotoxicity study or in the diet in the sub-chronic study. The NOAEL for the gavage study was 2 mg/kg/day and 7.99 mg/kg/day in males and 9.4 mg/kg/day in females in the sub-chronic study. Effects at the LOAEL were clinical signs and changes in field observation battery results. In the dietary study there were decreases in body weight, body weight gain and food consumption (EPA 2002d).

Cyhalothrin

Cyhalothrin, γ and λ isomers are synthetic pyrethroids registered for use on sweet corn under the brand names Proaxis (EPA# 74921-3) and Warrior (EPA# 100-1112), respectively (Table 13). The toxicity review below is from the 2002 tolerance petition (EPA 2002c). The later tolerance petitions, EPA 2004a and EPA 2008g, refer back to this review.

Sub-chronic Cyhalothrin Toxicity Studies

Sub-chronic (28 days to 6 months) feeding studies have been done with either cyhalothrin or λ -cyhalothrin in rats, mice and dogs. The NOAELs ranged from 1 mg/kg/day in the 28-day rat study to 77.9 mg/kg/day in female mice fed cyhalothrin for 28 days. The effects observed at the LOAELs included decreases in body weight gain, food consumption, food efficiency, clinical signs of neurotoxicity in the rats, increase in mortality, clinical signs of toxicity, and changes in hematology, organ weights and minimal liver enlargement in the mice, and increase in diarrhea in the dogs (EPA 2002c).

Twenty-one day dermal studies were done in the rabbit, using cyhalothrin and in the rat using λ -cyhalothrin. The NOAELs were 100 mg/kg/day in the rabbit and 10 mg/kg/day in the rat. Effects observed at the LOAELs were weight loss in the rabbits at 1,000 mg/kg/day and clinical signs of toxicity, decrease in body weight and body weight gain in the rats at 50 mg/kg/day.

Cyhalothrin Developmental Studies

The developmental studies were performed using cyhalothrin. The maternal NOAELs were 10 mg/kg/day in the rat and in the rabbit studies. The developmental NOAELs were 15 mg/kg/day in the rat and 30 mg/kg/day in the rabbit, in both cases the HDT. Effects in the dams at the LOAEL were uncoordinated limbs, decrease in body weight and decrease in food consumption (EPA 2002c).

Cyhalothrin Reproductive Studies

The reproduction studies were also performed using cyhalothrin. The results of the three generation rat study were a parental and offspring NOAEL of 1.5 mg/kg/day and a LOAEL of 5 mg/kg/day. Effects in the parental generation and the offspring at the LOAEL were decreases in body weight and body weight gain. There were no reported effects on reproductive parameters, therefore the NOAEL was 5 mg/kg/day the HDT (EPA 2002c).

Cyhalothrin Chronic-Oncogenicity Studies

Chronic studies performed in dogs (λ -cyhalothrin via capsules for 1 yr) resulted in a NOAEL of 0.1 mg/kg/day. Effects at the LOAEL were clinical signs of neurotoxicity. The combine oncogenicity/chronic studies in rats and mice were negative for oncogenic effects. The NOAELs for these studies were 2.5 mg/kg/day in the rat and 15 mg/kg/day in the mouse. Effects at the LOAEL were and decrease in body weight in the rat and increase in piloerection, hunched posture in mice. There was also a decrease in body weight gain seen in female mice (EPA 2002c).

The current EPA cancer classification for cyhalothrin is “D” not classifiable and for γ -cyhalothrin it is “not likely to be a human carcinogen” (EPA 2006b). EPA’s cancer classification criteria are found in Appendix D.

Esfenvalerate

Esfenvalerate is an enriched isomer of fenvalerate and is registered for use on sweet corn under the name Asana XL (EPA# 352-515) (Table 13).

Acute Esfenvalerate Toxicity Studies

The oral LD₅₀ for technical esfenvalerate is 87.2 mg/kg and the dermal LD₅₀ is greater than 2,000 mg/kg. Inhalation tests were waived due to the negligible vapor pressure of this esfenvalerate. It is a slight eye irritant, negative for primary skin irritation and is a non-sensitizer in guinea pigs (EPA 1998a).

Sub-chronic Esfenvalerate Toxicity Studies

Three sub-chronic studies using esfenvalerate were reviewed by EPA, in their 1998 tolerance action. Two 90-day dietary studies in rats and one subchronic feeding study in mice. The lowest NOAEL from these studies was 5 mg/kg/day in rats. Effects at the LOAELs were neurological in nature (EPA 1998a).

Esfenvalerate Developmental Studies

The rat and rabbit gavage studies with esfenvalerate resulted in a maternal NOAEL of 2

mg/kg/day with neurological, behavior and clinical signs observed at the LOAELs of 2.5 mg/kg/day and 3 mg/kg/day, respectively. The developmental NOAELs were greater than or equal to 20 mg/kg/day, the HDT in both studies (EPA 1998a).

Esfenvalerate Reproductive Studies

The 2-generation study in rats, the parental LOAEL was 3.75 mg/kg/day, LDT. The effect at this dose was a decrease in body weight. The NOAEL was not determined. The reproductive LOAEL was 5 mg/kg/day. Effects at this dose level were a decrease in pup body weight and a decrease in litter size. The reproductive NOAEL was 3.75 mg/kg/day (EPA 1998a).

Chronic-Oncogenicity Esfenvalerate Studies

The range finding (21-day) and 1-yr dog studies had LOAELs of 6.4 mg/kg/day and NOAELs of 2.25 and 5.29 mg/kg/day, respectively. The effects at the LOAEL in both these studies were an increase in neurological effects and decreases in body weights and food consumption (EPA 1998a).

Two supplement chronic-oncogenicity fenvalerate feeding studies in rats were reported. When both studies were taken together, EPA said that the guideline for combine chronic/ oncogenicity testing in rats was filled. The NOAEL in the first study was the HDT, 12.5 mg/kg/day. The second study used a dose of 50 mg/kg/day and the results at this dose were an increase in spindle cell sarcomas (later retracted by EPA), neurological effects and a decrease in body weight (EPA 1998a).

The chronic-oncogenicity mouse studies for fenvalerate included the 2 yr, 18 month and lifetime studies. The 2 yr study was met the guideline. The LOAEL was 7.5 mg/kg/day, with an increase in granulomatous changes (related to fenvalerate not esfenvalerate) observed. The NOAEL in this study was 1.5 mg/kg/day (EPA 1998a). Effects reported from the 18 month and the lifetime studies (both supplemental) in mice were increases in granulomatous changes and changes in hematology. EPA's current cancer classification for fenvalerate and esfenvalerate is "E" evidence of non-carcinogenicity in humans (EPA 2006b). EPA's cancer classification criteria are found in Appendix D.

Methomyl

Methomyl (CAS# 16752-77-5) is a member of the N-methyl carbamate (NMC) class of insecticides (EPA 1998b). It is the active ingredient in Lannate SP (EPA# 352-342) used on sweet corn in Maine (Table 13).

The mechanism of action for methomyl is reversible inhibition of cholinesterase (ChE) enzymes resulting at increases in acetylcholine (ACh) at neural and neural muscular junctions. The enzyme in the central nervous system is acetylcholinesterase (AChE) and in the blood. The enzymes in blood are butyrylcholinesterase (BChE) found in the plasma and AChE found in the

red blood cells (RBC). EPA published a Registration Eligibility Decision (RED) for Methomyl in 1998 (EPA 1998b), an updated the dietary risk assessment in 1997 (EPA 2007f) and included methomyl in the “*Revised N-Methyl Carbamate Cumulative Risk Assessment*” in 2007 (EPA 2007e). The studies in following toxicology review are from the 1998 RED (acute, sub-chronic, developmental and reproductive and chronic-oncogenicity studies) and the 2007 cumulative risk assessment (neurotox studies looking at cholinesterase inhibition).

Acute Methomyl Toxicity Studies

The methomyl oral LD₅₀s in male and female rats are 34 mg/kg and 30 mg/kg, respectively. The dermal LD₅₀ in rabbits is greater than 2,000 mg/kg. The 4-hr LC₅₀ in rats was 0.258 mg/L. Methomyl exposure causes corneal opacity and is negative for skin irritation, dermal sensitization and delayed neurotoxicity in hens. All of the methomyl containing products are commercial or industrial. The products, except for the 1% fly baits are restricted use, skull and cross bones due to acute toxicity concerns in mammals (EPA 1998b).

Sub-chronic Methomyl Toxicity Studies

The no observable effect level (NOEL) from the 90-day rat feeding study was 6.25 mg/kg/day and the lowest observable effect level (LOEL) was 12.5 mg/kg/day. Effects at the LOEL were a decrease in body weight (males and females) and an increase in erythroid hyperplasia in the bone marrow in males. The other sub-chronic study reported was a 90-day dog feeding study. This study rated as “unacceptable” to fulfill guideline requirements due to a lack of quality assurance and quality control (purity of the test substance was not reported, stability of the test compound was not demonstrated and the actual concentration of methomyl in the diet was not determined). In this study the reported NOELs were 14.68 mg/kg/day (males) and 12.5 mg/kg/day (females), in both cases the HDTs (EPA 1998b).

The 21-day dermal study in rabbits resulted in a NOEL of 5 mg/kg/day for cholinesterase inhibition, with a LOEL of 50 mg/kg/day. The systemic NOEL was 90 mg/kg/day (HDT). Clinical signs were present in some animals, but there was no dose response observed (EPA 1998b).

Methomyl Developmental Studies

Developmental toxicity studies reported in the methomyl 1998 RED include rat and rabbit studies. The NOELs for maternal effects were 9.4 mg/kg/day and 6 mg/kg/day, respectively. The LOEL for maternal toxicity in the rat study was 33.9 mg/kg/day (HDT). Effects at the LOEL were decreases in body weight and food consumption. The developmental NOEL in the rats was 33.9 mg/kg/day (HDT). The LOEL for maternal toxicity in the rabbit study was 16 mg/kg/day. Effects at the LOEL were increases in mortality and clinical signs. The developmental NOEL was 16 mg/kg/day (HDT) (EPA 1998b).

Methomyl Reproductive Studies

In the 2-generation rat reproduction study the parental NOEL was 3.75 mg/kg/day, with a LOEL of 30 mg/kg/day. Effects at the LOEL were decreases in body weight and food consumption with changes in hematology parameter. The NOEL and LOEL for the offspring were also 3.75 mg/kg/day and 30 mg/kg/day. Effects in the offspring at the LOEL were decreases in live pups and pup body weight (EPA 1998b).

The FQPA SF was reduced to 3X for methomyl. This factor is 10X, unless there is data indicating that the developing organism is no more sensitive than the adult. While this is the case with methomyl, EPA only reduced the FQPA SF to 3X because of a lack of acute and sub-chronic neurotoxicity studies. This safety factor is applied when EPA is assessing risks if pregnant women and/or young children are likely to be exposed, residential uses and diet/water chronic exposures.

Chronic-Oncogenicity Methomyl Studies

The chronic studies for methomyl include 2-yr rat, mouse and dog studies. These were all negative for oncogenic endpoints. The NOELs from the rat and dog studies were 5 mg/kg/day and 2.5 mg/kg/day respectively. Effects at the LOELs were a decrease in body weight gain in rats and changes in kidney histopathology in dogs. Neither the NOEL or LOEL were reported from the mouse study.

The latest EPA carcinogen classification for methomyl was “E” evidence of non-carcinogenicity (EPA 2006b). EPA’s cancer classification criteria are found in Appendix D.

EPA’s 2007 “Revised N-Methyl Carbamate (NMC) Cumulative Risk Assessment”

Methomyl is one of nine NMC insecticides evaluated by EPA in their 2007 cumulative risk assessment. EPA used the benchmark dose (BMD) approach, rather than the NOEL/LOEL approach used above in estimating risks in the cumulative risk assessment. This approach is described in detail in Appendix E.

EPA’s modeling from the NMC cumulative risk assessment resulted in a BMD₁₀ of 0.486 mg/kg/day for rat brain AChE inhibition and 0.204 mg/kg/day for red blood cell (RBC) AChE inhibition. Comparing this to BMD₁₀ of 0.040 mg/kg/day for RBC AChE inhibition from the human study the resulting interspecies extrapolation factor is 5X (ratio of BMD₁₀s for RBC AChE inhibition in rats to humans; $0.204/0.040 = 5.1$) (EPA 2007e).

EPA based the FQPA SF of 3X for methomyl on the ratio of the BMD₁₀ for inhibition of whole brain AChE in adults to the BMD₁₀ for inhibition of whole brain AChE in pups at post natal day (PND11). In the case of methomyl the BMD₁₀ in adults was 0.317 mg/kg/day and that in pups PND11 was 0.104 mg/kg/day (ratio = 3.05).

These details are summarized from EPA tolerance documents with the exception of methomyl.

Methomyl is one of nine NMC insecticides evaluated by EPA in their 2007 cumulative risk assessment. EPA used the benchmark dose (BMD) approach, rather than the NOEL/LOEL approach used above in estimating risks in the cumulative risk assessment.

The synthetic pyrethroids are less acutely toxic in mammals than methomyl, from a chronic perspective these compounds have NOAELs of less than 6 mg/kg/day in dogs and between 2.5 and 12.5 mg/kg/day in rats (EPA 2007e).

Summary

Use of Bt sweet corn will decrease the amount of chemical pesticides used by sweet corn growers in Maine. The potential reduction in chemical pesticide use in sweet corn is summarized in Table 15. These reductions are limited to those insecticides used on ECB, FAW and CEW. The most significant reduction in chemical pesticide use is expected to be decreases in Lannate uses. If 100% of the sweet corn growers adopted the technology there would be a reduction of between 3,375 lbs and 9,450 methomyl (ai), typical and maximum use rates respectively. The use Bt sweet corn will reduce, but not eliminate the use of chemical insecticides (UMCE 1996).

Estimate of acreage from Technical Committee Report (BPC 2008) the 1400 acres treated with pyrethroids (280 acres/ pyrethroid) was equally distributed between the 5 active ingredients

Mycotoxins levels in Bt corn compared to non-Bt-corn

There were two primary sources for information on mycotoxins in Bt vs non-Bt corn. There were a number of studies designed to determine mycotoxins levels in corn under natural conditions, where the corn plants were manually infested with ECB, Southwestern Corn Borer (SWCB), CEW, and FAW and studies where the corn was infected by hand with the mycotoxins producing plant pathogen, *Fusarium*. In one study Bt-corn and its isoline were infested by hand with ECB and the plants were inoculated with *Fusarium* (Clements *et al.*, 2003).

In the other type of study, mycotoxins analysis was done to assure that the levels of mycotoxins would not confound effects of the Bt proteins in the diets.

Studies designed to analyze for mycotoxins residues

In the majority of trials (sets of Bt vs isoline controls) regardless of the source of fungi and insect larvae, there were significantly lower levels of mycotoxins in corn from the Bt lines. In one study, in one trial the DON levels were higher in the Bt-corn than the isoline (Bakan *et al.*, 2002). In the rest of the trials there were no statistically significant differences between the two groups (Table 17).

Over all conclusions

It was the consensus of the Medical Advisory Committee that the data required by EPA for registration of Bt corn products for application in sweet corn is inadequate to perform a human health risk assessment. It was also agreed that there is a relative absence of non-industry controlled safety data on Bt foods re: human health—the MAC would support the generation of such data prior to further registrations

Table 1. EPA Toxicity Categories 40 CFR 156.52				
Hazard indicators	Categories			
	I	II	III	IV
Oral LD₅₀	> 50 mg/kg	50 to 500 mg/kg	500 to 5,000 mg/kg	> 5,000 mg/kg
Inhalation LD₅₀	< 0.2 mg/L	0.2 to 2 mg/L	2 to 20 mg/L	> 20 mg/L
Dermal LD₅₀	< 200 mg/kg	200 to 2,000 mg/kg	2,000 to 20,000 mg/kg	> 20,000
Eye effects	Corrosive: corneal opacity not reversible within 7 days	Corneal opacity reversible within 7 days	No corneal opacity; irritation reversible within 7 days	No irritation
Skin effects	Corrosive	Severe irritation at 72 hrs	Moderate irritation at 72 hrs	Mild or slight irritation at 72 hrs

Study Event	Table 2. Summary of Differences Between Cry1Ab Bt-corn (Bt), Isolines and Commercial Dietary Parameters in Poultry Studies				
	Diet guidelines	Proteins; Carbohydrates; Fats	Pesticides	Mycotoxins	Diets
Rossi <i>et al.</i> , 2005 MON 810	Ross Breeder's Netherland 1989	No Differences	Negative	76% lower Fumonisin B ₁ in Bt diet	3 Bt /isoline
Taylor <i>et al.</i> , 2003a MON 810	National Research Council (NRC) 1994	No Differences	Negative	Negative	1 starter Bt/isoline 1 grower/finisher Bt/isoline 6 starter commercial 6 grower/finisher commercial
Taylor <i>et al.</i> , 2003b MON 810	NRC 1994	No Differences	Negative	Negative	2 starter Bt/isoline 2 grower/finisher Bt/isoline 4 starter commercial 4 grower/finisher commercial
Aeschbacher <i>et al.</i> , 2005 Bt176	NRC 1994	CP was higher in 1 diet; no effect was seen	Negative	Negative	1 Bt/isoline
Brake <i>et al.</i> , 2005	NRC 1994	CP and moisture were higher in North Carolina commercial (NC com) than the other diets varieties equalized in the diet	Not Reported	Fumonisions 8.8 to 20.6 ppm	Bt11-Liberty link (LL) Bt11-LL + Glufosinate-ammonium isoline NC com

Study Event	Table 3. Summary of Results of Cry1Ab Bt-corn (Bt), Isolines and Commercial in Poultry Studies					
	Feed Intake	Feed Efficiency	Body Weight	Organ or Tissue Weight	Meat Composition	Diets
Rossi <i>et al.</i> , 2005 MON 810	Negative	Negative	Negative	Negative	Not Reported	3 Bt /isoline
Taylor <i>et al.</i> , 2003a MON 810	Negative	Negative	Negative	Increase breast weight in combined sexes	Negative	1 starter Bt/isoline 1 grower/finisher Bt/isoline 6 starter commercial 6 grower/finisher commercial
Taylor <i>et al.</i> , 2003b MON 810	Negative	Negative	Negative	Negative	% protein decreased in breast meat Bt vs iso; Bt vs one com	2 starter Bt/isoline 2 grower/finisher Bt/isoline 4 starter commercial 4 grower/finisher commercial
Aeschbacher <i>et al.</i> , 2005 Bt176	Negative	Negative	Negative	Not reported	Not Reported	1 Bt/isoline
Brake <i>et al.</i> , 2005 Bt11	Not Reported	Increase NC com vs isoline NC com vs Bt groups	Decrease NC com vs isoline NC com vs Bt groups	Negative	Not Reported	Bt-Liberty link (LL) Bt-LL + Glufosinate-ammonium isoline NC com

Table 4. Isoline Groups vs Bt Groups, Summary of Statistically Significant Results in Male Rats from the Rat MON 863 diet study (Hammond *et al.*, 2006)

Bt Group	Parameter	Isoline +/- 1 SD	Bt-group +/- 1 SD	Bt vs Isoline P value
11%	Na+	153 +/- 2.5	150 +/- 0.09	< 0.01
33%	WBC	8.64 +/- 2.24	10.4 +/- 1.57	< 0.01
33%	LYM	7.21 +/- 2.30	8.80 +/- 1.48	< 0.01
33%	Baso	0.00 +/- 0.00	0.03 +/- 0.05	< 0.01
33%	APPT(s)	19.8 +/- 0.94	20.0 +/- 0.92	< 0.01
33%	Cl-	107.5 +/- 1.7	104.8 +/- 2.2	< 0.05

Table 5. Isoline vs Bt-Groups, Summary of Statistically Significant Results in Female Rats from the Rat MON 863 diet study (Hammond *et al.*, 2006)

Bt Group	Parameter	Isoline +/- 1 SD	Bt-group +/- 1 SD	Bt vs Isoline P value
11%	A/G	2.33 +/- 0.27	1.91 +/- 0.26	< 0.01
11%	GLOB	2.22 +/- 0.25	2.56 +/- 0.31	< 0.01
11%	PT (s)	14.9 +/- 0.42	15.4 +/- 0.20	< 0.05
11%	TRIG	40.9 +/- 12.3	50.9 +/- 7.8	< 0.01

Table 6. Bt-groups vs Reference Group; Summary of Statistically Significant in Female Rats Results from the Rat MON 863 diet study (Hammond *et al.*, 2006)

Bt Group	Parameter	Bt mean +/- 2 SD	Reference mean +/- 2 SD	Estimate of Reference Range (2 SD)	P value
11%	GLUC	113 +/- 11	115 +/- 22	93-137	< 0.05
33%	GLUC	116 +/- 8	115 +/- 22	93-137	< 0.05
33%	APPT(s)	16.5 +/- 1.1	20 +/- 4.80	15.2 - 24.8	< 0.01
33%	AST	94.8 +/- 12	89 +/- 56	33 – 145	< 0.05
33%	Ca ⁺⁺	11.2 +/- 2.8	11.5 +/- 1.3	24.3- 54. 7	< 0.05
33%	Cl ⁻	108.6 +/- 2.0	104 +/- 4.8	99.2-108.8	< 0.01
33%	TRIG	46.7 +/- 15.0	39.5 +/- 15.2	24.3-54.7	< 0.01

Table7. Percent Corn in the Swine Diet Hyun *et al.*, 2005

Stage	Weight kg	Study I	Study II
Grower I	22.7-43.5	68.65 %	65 %
Grower II	43.5-69.3	74.79 %	
Finisher I	69.3-98	76.66 %	72 %
Finisher II	98-117.2	82.47 %	76 %

Table 10. Bt Group (33 %) vs Isoline, Summary of Statistically Significant and Relevant Results in Male Rats from the Rat DAS-01507-1 (Cry1F) diet study (MacKenzie <i>et al.</i>, 2007)				
Parameter	Isoline +/- SD	Bt 33% +/- SD	Commercial corn +/- SD	Bt vs isoline P value
Feed Intake post wk 3	25.7 +/- 1.7	27.5 +/- 2.6	ND	< 0.05
Final BW	4.1 % increase over commercial	5.3 % increase over commercial	ND	< 0.05
Overall feed efficiency (g body wt/g feed consumed)	No difference	No difference	ND	ns
ALP	112 +/- 25	91 +/- 19	91 +/- 14	< 0.05
Relative kidney wt	0.753 +/- 0.076	0.0685 +/- 0.073	0.0750 +/- 0.057	< 0.05
Absolute kidney wt	No difference	No difference	ND	ns

Table 11. Bt Group (33%) vs Isoline, Summary of Statistically Significant and Relevant Results in Female Rats from the Rat DAS-01507-1 (Cry1F) diet study (MacKenzie <i>et al.</i>, 2007)					
Parameter	Isoline +/- SD	Bt 33% +/-SD	Commercial +/-SD	Commercial range	Bt vs Isoline P value
RBC	8.46 +/- 0.35	8.17 +/- 0.26	8.40 +/- 0.41	7.99-8.81	< 0.05
HCT	46.8 +/- 1.5	45.4 +/- 1	45.8 +/- 1.5	44.3-47.3	< 0.05
AEOS	0.13 +/- 0.06	0.11 +/- 0.03	0.16 +/- 0.07	0.09-0.23	< 0.05

Diet	Cry34Ab1			Cry35Ab1		
	Nominal	TWA Males	TWA Females	Nominal	TWA Males	TWA Females
PMI 5002	0	0	0	0	0	0
PMI 5002 with BSA	0	0	0	0	0	0
Low	1.97	1.84	2.13	0.078	0.073	0.085
Medium	19.7	18.4	21.3	0.78	0.73	0.85
High	197	184	213	7.8	7.3	8.5

Product	EPA #	Active Ingredients	Signal word	RUP; Reason	Lbs ai		Reference
					Acre	Acre/yr	
Synthetic Pyrethroids							
Asana XL	352-515	8.4% esfenvalerate	Warning	Yes, Aquatic Toxicity	0.05	0.5	DuPont 2007
Baythroid 2E	264-745	25% cyfluthrin	Danger	Yes, Aquatic Toxicity	0.044	0.44	Bayer 2007a
Baythroid XL	264-840	12.7% β -cyfluthrin	Warning	Yes, Aquatic Toxicity	0.022	0.22	Bayer 2007b
Warrior	100-1112	11.4% λ -cyhalothrin	Warning	Yes, Aquatic Toxicity	0.03	0.48	Syngenta 2007
Proaxis	74921-3	5.9% γ -cyhalothrin	Caution	Yes, Aquatic Toxicity	0.015	0.24	Pytech 2005
Carbamate							

Table 13. Sweet Corn Insecticides Used on Sweet Corn in Maine ^(a)							
Product	EPA #	Active Ingredients	Signal word	RUP; Reason	Lbs ai		Reference
					Acre	Acre/yr	
Lannate	352-342	90% methomyl	Danger; Skull and Cross bones	Yes, Human Toxicity	0.45	6.3	DuPont 2008

Table 14. Estimates of Chemical Pesticides which Would Not Be Used if Bt Sweet Corn Were Planted						
Active Ingredients (ai)	MAX Acres ^(a)	Lbs ai /Acres	# Treatments/ year ^(b)		Statewide lbs ai/ year ^(b)	
			Label	Typical	Label	Typical
Esfenvalerate	280	0.05	10	5	140	70
Cyfluthrin	280	0.044	10	5	123	62
β -cyfluthrin	280	0.022	10	5	62	31
λ -cyhalothrin	280	0.048	16	5	134	42
γ -cyhalothrin	280	0.024	16	5	67	21
Methomyl	1500	0.45	14	5	9450	3375

Table 15 Composition of Cyfluthrin and β-Cyfluthrin [(RS)-α-cyano-4-fluoro-3-phenoxybenzyl (1R, 3R; 1R, 3S)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate] (CAS# 68359-37-5) (EPA 2007a)			
#	Diastereomeric Pair Stereochemistry	% in Technical grade	
		Cyfluthrin	B-Cyfluthrin
I	1R, 3R, α R + 1S, 3S, α S; 1:1; cis	23 to 27%	< 2 %
II	1R, 3R, α S + 1S, 3S, α R; 1:1; cis	17 to 21%	30 to 40 %
III	1R, 3S, α R + 1S, 3R, α S; 1:1; trans	32 to 36%	<3 % %
IV	1R, 3S, α S + 1S, 3R, α R; 1:1; trans	21 to 25 %	53 to 67 %

Table 16 Summary of Toxicity Databases for Chemical Insecticides Used on Sweet Corn in Maine								
Compound	Oral LD ₅₀ (mg/kg) ^(a)	NOAELs (mg/kg/day) ^(b)					FQPA SF ^(f)	Cancer Classification ^(g)
	Rat	Developmental ^(c)		Reproduction ^(d)		Chronic ^(e)		
		Dams	Pups	Parents	Pups			
Cyfluthrin	500 to 900 ^(a)	3	10	3	9	2.43	1X	Not likely
Cyhalothrin	50 to 166	10	15	1.5	5	0.1	1X	D
Esfenvalerate	75 to 88	2	<20	ND	3.75	5.29	1X	E
Methomyl	30 to 34 ^(b)	6	16	3.75	3.75	2.5	3X	E

(a) Range of LD50s from the Pesticide Manual (2003)

(b) Lowest No Observable Adverse Effect Levels (NOAELs)

(c) NOAELs from gavage, rat or rabbit, developmental studies

(d) NOAELs from multigenerational feeding studies

(e) NOAELs from 1-yr dog or 2-yr rat feeding studies

(f) Food Quality protection Act Safety Factor (FQPA SF) as assigned by EPA or estimated based on the developmental and reproduction toxicity studies

(g) EPA Cancer Classification from EPA 2006b (see Appendix D)

Table 17. Summary of Bt-corn vs Isoline Studies for Mycotoxin Analysis

Mycotoxin	Bt vs Isoline	# Trials	Conditions	Study
3-A-Deoxynivalenol	Decrease	1	Manual Infestation ECB	Papst <i>et al.</i> , 2005
Aflatoxins	No Difference	1	Manual Infestation ECB, SWCB, CEW, FAW	Hammond <i>et al.</i> , 2004
Aflatoxins	No Difference	1	Natural	Hammond <i>et al.</i> , 2004
Deoxynivalenol	No Difference	1	Manual Infestation ECB	Papst <i>et al.</i> , 2005
Deoxynivalenol	No Difference	1	Manual Infestation ECB, Insecticide protected	Papst <i>et al.</i> , 2005
Deoxynivalenol	No Difference	1	Manual Infestation ECB, SWCB, CEW, FAW	Hammond <i>et al.</i> , 2004
Deoxynivalenol	Decrease	3	Natural	Bakan <i>et al.</i> , 2002
Deoxynivalenol	Increase	1	Natural	Bakan <i>et al.</i> , 2002
Deoxynivalenol	No Difference	2	Natural	Hammond <i>et al.</i> , 2004, Bakan <i>et al.</i> , 2002
Fumonisin	Decrease	12	Manual Infestation ECB	Clements <i>et al.</i> , 2003, Papst <i>et al.</i> , 2005
Fumonisin	Decrease	2	Manual Infestation ECB, SWCB, CEW, FAW	Hammond <i>et al.</i> , 2004
Fumonisin	No Difference	4	Manual Inoculation <i>Fusarium</i>	Clements <i>et al.</i> , 2003

Table 17. Summary of Bt-corn vs Isoline Studies for Mycotoxin Analysis

Mycotoxin	Bt vs Isoline	# Trials	Conditions	Study
Fumonisin	Decrease	4	Manual Inoculation <i>Fusarium</i> and Infestation with ECB	Clements <i>et al.</i> , 2003
Fumonisin	Decrease	20	Natural	Hammond <i>et al.</i> , 2004, Bakan <i>et al.</i> , 2002, de la Camp <i>et al.</i> , 2005, Clements <i>et al.</i> , 2003, Dowd <i>et</i> <i>al.</i> , 2001
Fumonisin	No Difference	10	Natural	Hammond <i>et al.</i> , 2004, de la Camp <i>et al.</i> , 2005, Dowd <i>et al.</i> , 2001
Moniliformin	Decrease	1	Manual Infestation ECB	Papst <i>et al.</i> , 2005
Nivalenol	Decrease	1	Manual Infestation ECB	Papst <i>et al.</i> , 2005
Nivalenol	Decrease	2	Natural	Bakan <i>et al.</i> , 2002
Nivalenol	No Difference	3	Natural	Bakan <i>et al.</i> , 2002
Zearalenone	Decrease	1	Natural	Bakan <i>et al.</i> , 2002
Zearalenone	No Difference	4	Natural	Bakan <i>et al.</i> , 2002

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