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An overview of the safety and biological effects of *Bacillus thuringiensis* Cry toxins in mammals

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ABSTRACT: Crystal proteins (Cry) produced during the growth and sporulation phases of *Bacillus thuringiensis* (Bt) bacterium are known as delta endotoxins. These toxins are being used worldwide as bioinsecticides to control pests in agriculture, and some Cry toxins are used against mosquitoes to control vector transmission. This review summarizes the relevant information currently available regarding the biosafety and biological effects that Bt and its insecticidal Cry proteins elicit in mammals. This work was performed because of concerns regarding the possible health impact of Cry toxins on vertebrates, particularly because Bt toxins might be associated with immune-activating or allergic responses. The controversial data published to date are discussed in this review considering earlier toxicological studies of *B. thuringiensis*, spores, toxins and Bt crops. We discussed the experimental studies performed in humans, mice, rats and sheep as well as in diverse mammalian cell lines. Although the term 'toxic' is not appropriate for defining the effects these toxins have on mammals, they cannot be considered innocuous, as they have some physiological effects that may become pathological; thus, trials that are more comprehensive are necessary to determine their effects on mammals because knowledge in this field remains limited. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: *Bacillus thuringiensis*; Bt-derived bioinsecticides; transgenic plants; cry toxins; mammals biosafety; biological effects; immunological effects; allergenicity

Considerations of the selection criteria for inclusion used in this review

The present work is a narrative review; however, because of the sensitivity of this discussion topic, which can be affected by financial or ideological interest conflicts, we have indicated some of the selection criteria for inclusion used in this review. We are aware that these criteria are normally deeply defined for elaborate systematic reviews while we are briefly mentioning them here. The purpose of this work was to conduct an unbiased review of the currently available information regarding the safety of the biological effects of Bt and its insecticidal Cry proteins on mammals. Thus, we searched for every available evidence stream indicating either the effects or safety of these proteins in mammals based on exhaustive searches of reports retrieved from diverse academic databases. We included both information recorded from primary academic peer review reports and regulatory studies, but the last sources are categorized in the reference section. We also categorized reports published on behalf of any GM company due to the implicit existing financial conflict of interest.

Primary peer-reviewed and regulatory studies were found by searching in PubMed, Google Scholar and Scopus using diverse keywords related to the safety, effects or toxicity of Bt or Cry proteins. We also used related item searching criteria to retrieve additional information. We searched for every experimental academic work reported at any time in which any effect of Cry toxins or Bt crops on mammals had been reported. Then, we selected the reports to review their contents and to cite them according to their relevance. Furthermore, we reviewed literature in which *in vitro* assays were performed to test the cytotoxicity of Cry toxins on different cell lines.

We included the primary four review papers referring to the safety of Bt products that have been cited in the majority of experimental works. These primary reviews were performed by McClintock *et al.* (1995), Siegel (2001); Betz *et al.* (2000) and, most recently, by Koch *et al.* (2015). We critically analysed the information summarized in those works along with data from the original experimental studies cited in those reviews and discussed them together with the information obtained from the US Environmental Protection Agency (EPA) that was used to approve the use of Bt products (McClintock *et al.*, 1995; Betz *et al.*, 2000; Siegel, 2001; Koch *et al.*, 2015).

We analysed the studies conducted by the Monsanto Company, the principal transgenic crop producer that has evaluated and approved the safety of these crops. We discussed the weak issues, and the missing studies that we consider remain needed to demonstrate clearly the safety or the absence of any biological effect of Cry proteins on mammals.

We selected the few reports that demonstrated any effect of Bt crops, including the first test performed in humans, and we discussed the epidemiological studies performed in Bt-exposed populations and the three cases of unique infections in humans caused by Bt.

Finally, we presented and discussed the immunostimulating effects of purified Cry toxins reported by different laboratories and data related to the immunogenic and adjuvant effects of the Cry1Ac protoxin, which have been reported by our group in the last 15 years.

Finally, we declare that no conflicts of interest exist.

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Introduction

Bacillus thuringiensis (Bt), which was discovered in 1901 by Ishiwata Shigetane, is the causal agent of a silkworm disease, this Gram-positive bacterium produces proteinaceous body inclusions (Whiteley and Schnepf, 1986) known as Cry proteins or δ-endotoxins during its sporulation phase. Cry proteins are produced as protoxins of 70–130 kDa and require proteolytic activation to generate Cry toxin fragments of 60–65 kDa that are toxic to a wide variety of important agricultural and health-related insects. The activated toxin interacts with the midgut epithelium of susceptible insects, generating pores in the membrane that consequently kill the specific insect target (Hofte and Whiteley, 1989). Commercial insecticides based on the bacterium Bt were registered in the United States in 1961 because of their effectiveness, high specificity and environmental safety (Siegel, 2001).

For these advantageous features, Cry proteins are an important alternative to chemical pesticides for controlling insect pests in crops, forests and homes. Additionally, Cry toxins have been expressed in transgenic plants, providing a powerful method for protecting crops against insect damage (Barton *et al.,* 1987; Vaeck *et al.,* 1987). The first commercialization of transgenic crops expressing Cry toxins from Bt occurred in 1996; from then on, worldwide production of these crops has increased dramatically. In 2003, six transgenic crops, corn, papaya, canola, soybean, cotton and squash, were included. These additional crops improved farm income by US $1.9 billion, and the initial cultivation hectares (ha) increased dramatically from approximately 1.7×10^6 ha to more than 6.6×10^7 ha in 2011 (Li *et al.,* 1991, 2013).

Several studies have proven that Bt insecticides are safe for vertebrates at several thousand-fold doses higher than those expected to be found in the environment or transgenic plants (McClintock *et al.*, 1995). Nevertheless, most studies have analysed acute, but not chronic, effects, as only a few studies have been published regarding the effects of mid- or long-term exposure to Bt insecticides. A notable study in humans highlighted the presence of Cry1Ab in maternal, foetal and nonpregnant women's blood following exposure to Cry1Ab. As this toxin was clearly detectable and was able to cross the placenta to the foetus (Aris and Leblanc, 2011). Moreover, the direct effects of Cry toxins on mammalian cells have not yet been fully studied. The possible health risks of consumption of genetically modified organisms (GMOs) is under controversial debate worldwide (Konig *et al.,* 2004; Seralini *et al.,* 2009). Doubts regarding the safety of GMOs are encouraged because of the presence of conflicts of interest with some of these studies, particularly with the study conducted under the responsibility of the Monsanto Company regarding the transgenic corn MON863 that was finally approved in 2005 (Seralini *et al.,* 2007).

In the first part of this review, we present general aspects of Cry toxins such as the nomenclature and action mechanisms in target insects. Then, we review the toxicological studies of Bt bioinsecticides performed in mammals and the current knowledge regarding the safety, risks and effects of Cry toxins on mammals using a biotechnological approach. We also show an overview of the toxicological analysis of bioinsecticides including the complete Bt bacterium, spores, toxins and Bt transgenic plants. We presented a global view in the context of studies performed on humans, animals and cell lines. Taken together, the revised results indicate that Bt-Cry toxins are safe but are not innocuous because our studies and those of others researchers had showed effects on either the immune system or cell viability and/or possible allergenic effects.

Nomenclature and structure of Cry toxins

Bt δ -endotoxins include Cry and Cyt proteins, which are classified by their primary amino acid sequences and which are divided into four phylogenetically non-related protein families with different modes of action (Bravo *et al.,* 2011): the Cry family toxins (three domain toxins), the Cyt family toxins, the Mtx family (mosquitocidal Cry toxins) and the Bin family (binary-like) (Estruch *et al.,* 1996; Warren, 1997; Bravo *et al.,* 2004).

To date, the Cry proteins are distributed into 50 groups with more than 200 different gene sequences. The largest Cry family has three domains, and different Cry toxins have been resolved by crystallography including Cry1Aa, Cry2Aa, Cry3Aa, Cry3Ba, Cry4Aa, Cry4Ba and Cry8E (Li *et al.,* 1991; Grochulski *et al.,* 1995; Galitsky *et al.,* 2001; Morse *et al.,* 2001; Boonserm *et al.,* 2006; Guo *et al.,* 2009). The nomenclature of Cry1 toxins includes an Arabic number (for example, Cry1 and Cry2) that corresponds to 45% of identity between toxins. The second position (a capital letter) corresponds to 45% to 78% of identity between toxins (for example, Cry1A and Cry1B). Finally, a lowercase letter corresponds to 78% to 95% of identity between. The final nomenclature is Cry1Aa, Cry1Ab, Cry1Ac, and others.

Cry toxins are composed of three domains. Domain I consists of seven antiparallel amphipathic alpha helices, six of which surround helix number 5. This domain is implicated in membrane insertion, toxin oligomerization and lytic pore formation. Domain II is composed of a beta-prism of three anti-parallel beta-sheets with exposed loop regions; this domain is the less conserved in sequence and is involved in receptor recognition. Domain III, is a beta-sandwich of two antiparallel beta-sheets. Both domains II and III are implicated in insect specificity and interact with different insect midgut proteins (Li *et al.,* 1991; Grochulski *et al.,* 1995; Galitsky *et al.,* 2001; Morse *et al.,* 2001; Boonserm *et al.,* 2005, 2006; Bravo *et al.,* 2004, 2007, 2011).

Action mechanism of Cry toxins in insects

Cry proteins are produced as crystal protoxins in the parasporal inclusion bodies of Bt; when these protoxins are ingested by susceptible larvae, they are first solubilized in the midgut because of the extreme alkaline pH conditions and then are proteolytically processed. After the proteolytic activation of the protoxin by midgut proteases, Cry toxins bind to the surface proteins in the larvae midgut cells and form pores (Aronson and Shai, 2001; Bravo *et al.,* 2004). The binding proteins for Cry1 toxins have been determined in lepidopteran insects and include cadherin-like receptor (CADR), glycosylphophatidyl inositol (GPI)-anchored aminopeptidase-N (APN), GPI-anchored alkaline phosphatase (ALP), a 270-kDa glycoconjugate and a 250-kDa protein named P252 (See Box 1 and reviewed by Pigott and Ellar, 2007). Once the Cry toxin binds in its monomeric form to the CADR receptor, additional proteolytic cleavage of the toxin is promoted where the alpha-1 helix is eliminated. Then, hydrophobic residues exposed in the medium lead to the formation of the oligomeric pre-pore, which may have a tetrameric structure and a weight of 250 kDa (Gomez *et al.,* 2007). The oligomeric pre-pore forms stable channels with an open configuration (Rausell *et al.,* 2004) that allows more efficient insertion into the membrane compared with the monomeric toxin. In the case of Cry1Ab, the oligomeric pre-pore has a 200-fold higher affinity (0.75 nM Kd) to APN receptor than the monomeric structure of the toxin (100 nM Kd).

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The binding to the second receptor, GPI-anchored APN, results in toxin insertion into lipid rafts causing osmotic lysis and subsequent insect death (Atsumi *et al.,* 2005). Figure 1 illustrates the activation and action mechanisms of Cry1A toxins in insects: (i) shows a diagram of the Cry1A protoxin structure, indicating the protease cleavage sites to generate the activated toxin and (ii) summarizes the schematic mode of action of Cry1A on insects (Bravo *et al.,* 2004; Gomez *et al.,* 2007). In addition to the mode of action described above, Zhang *et al.* (2006) proposed that Cry protein toxicity could be related to G-protein mediated apoptosis after receptor binding; nevertheless, additional studies are needed to examine this hypothesis (Zhang *et al.,* 2006).

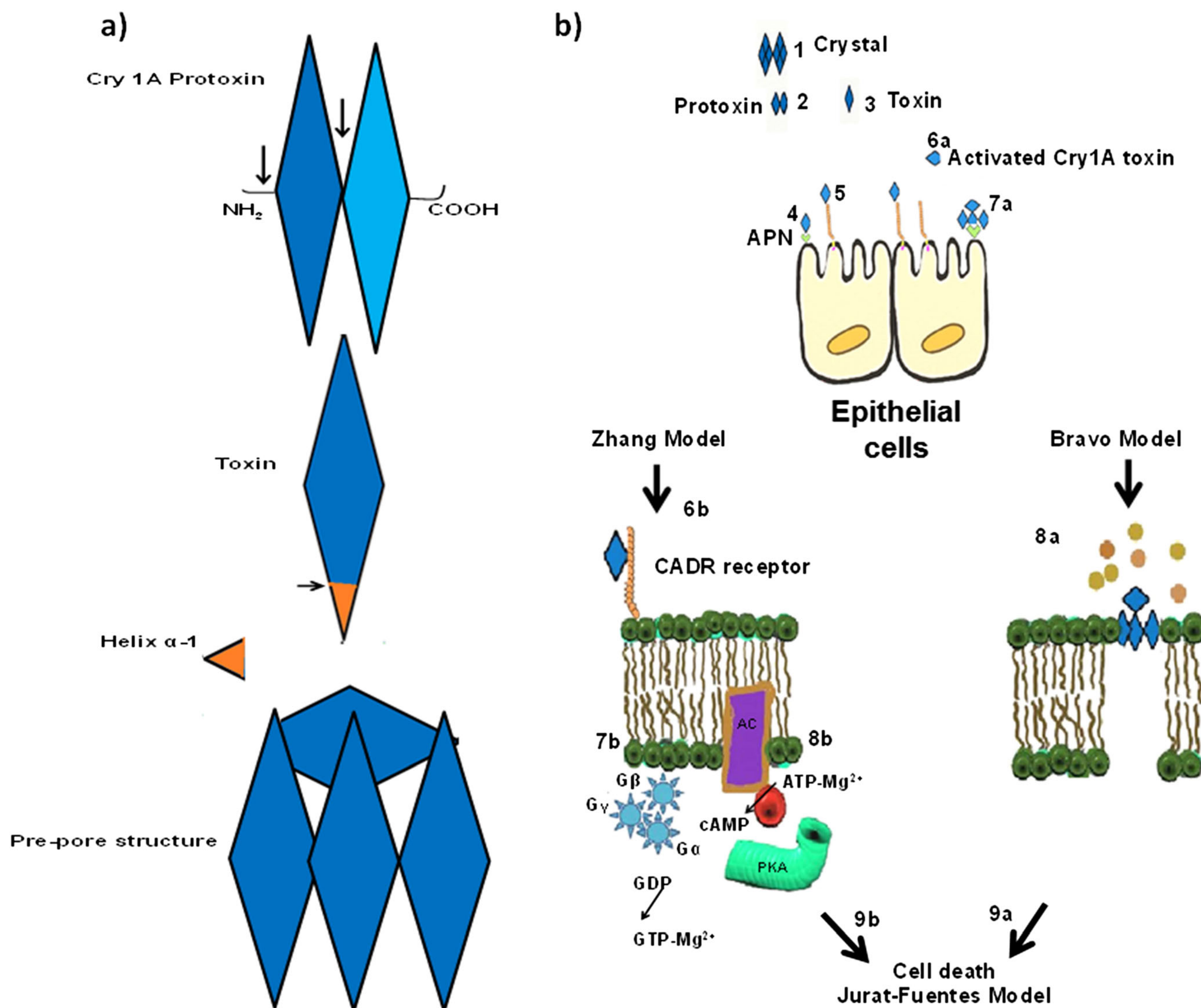

Figure 1. Activation and action mechanism of Cry1A toxins in insects. (A) Proteolytic digestion of Cry1A protoxins is required to form the pre-pore structure. The Cry1A protoxin is represented by two blue rhombuses (light and dark), and a single rhombus represents the activated toxin. Solid arrows show the cleavage sites to remove approximately half of the protein from the C-terminus. The cleavage of the N-terminal peptide approximately 25–30 amino acids (orange triangle) in Cry1A protoxins is also needed to obtain the active toxin. This proteolytic cleavage occurs after the toxin binds with cadherin receptor in the insect midgut epithelia and removes the helix α-1 in domain I. Finally, the pre-pore structure is assembled. (B) Action mechanism of Cry1A toxins in insects. 1) Crystals are ingested by susceptible insects, for example, *Manduca sexta.* 2) Solubilization is performed in midgut cells. 3) The active toxin is obtained through proteolytic processing in the insect midgut. 4) Activated Cry1A binds in monomeric form to highly abundant GPI-anchored aminopeptidase-N (APN) with low affinity and to alkaline phosphatase (ALP). 5) In addition to binding with the APN receptor, binding of monomeric Cry1A toxin with cadherin receptor also occurs. 6a) The Bravo model proposes that the union with the cadherin receptor allows the loss of α-helix 1 in domain 1 of Cr1A toxin. 7a) Oligomeric structure binds with high affinity to GPI-anchored N-aminopeptidase. 8) The insertion into membrane of the oligomeric Cry1A is performed causing an osmotic shock 9a) that culminates with the insect death. In this model, both cadherin and APN receptors are required for full Cry1A toxicity. The second model to describe the toxicity of Cry1Ab toxin was proposed by Zhang *et al.* (2006). 6b) These authors proposed a new model of action for Cry1Ab in which monomeric Cry1Ab binds to cadherin (CADR) receptors and initiates Mg^{2+}-dependent signalling. 7b) These authors suggested that receptor binding activates a signalling pathway involving stimulation of G protein and adenylyl cyclase (AC), increased cyclic AMP (cAMP) levels, and activation of kinase A (PKA). 8b) The PKA pathway generates cytological changes that include membrane structure, appearance of ghost nuclei, cell swelling, and lysis. 9b) The Jurat–Fuentes model suggests that cytotoxicity is due to the combined effects of osmotic lysis and cell signalling and thus implies the participation of elements of both the Bravo and the Zhang models.

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The expansion of Cry bioinsecticides: from Bt sprays to GM Bt crops

The first commercialization of Bt insecticides was made in France in the late 1930s. Since that decade and over the next 60 years, Bt has become one of the most significant biopesticides containing a mixture of spores and insecticidal crystals. The Organization for Economic Cooperation and Development (OECD) predicted that this biopesticide may increase to 20% of the world's pesticide market by 2020 (Whalon and Wingerd, 2003). Sprayable Bt formulations are typically used over small areas where cotton, fruits and vegetables are grown.

However, the spray Bt insecticides have several disadvantages. These insecticides cannot be applied uniformly to all parts of the plant or delivered to pests that are inside plant tissues. Additionally, the exposition period of their bioinsecticidal activity is low, as Bt spores are susceptible to rapid degradation by UV light and removal by water runoff.

Generally, it has been argued that the disadvantages of spray Bt bioinsecticides can be partially abolished with the generation of GM Bt crops, which would also be presumed to be safe, that is, to lack harmful effects on vertebrates and humans and to have low impacts on nontarget organisms due to the narrow existing spectrum of primarily leaf-feeding lepidopteran targets. Based on these advantages, transgenic plants encoding these insecticidal proteins have been generated throughout the world via recombinant DNA technology.

Since the introduction of Bt-protected crops such as corn and cotton in 1995/1996, these crops have had provided superior protection against insects compared to that shown by conventional chemical pesticides (Betz *et al.,* 2000). The global area occupied by transgenic crops has increased notably since 1996 to 2014, from 1.7 to 181.5 million ha in 28 countries according to the ISAAA (Navarro, 2015). In 2011, transgenic maize was the second most important GM crop after soybean, occupying 32% of the global area of GM crops (James, 2011). Many crops expressing distinct Cry toxins have been approved for commercial use (depicted in Table 1). As a result of the economic impact that these bioinsecticides have on agriculture, we feel that it is important to review the current status of safety and effects of *Bacillus thuringiensis* (Bt) derivates first and then to discuss the toxicological studies performed with GM transgenic plants. This distinction was made because, as discussed later, Bt toxins produced by GM organisms can be considered modified Bt toxins.

Toxicological studies of Bt-derived bioinsecticides: *Bacillus* and spores

Cry proteins are considered safe based on the following three features: these proteins are generally not toxic to mammals, they are digestible, and they do not bioaccumulate in fat tissues. Cry toxins are considered environmentally friendly, because they affect relatively few insect species due the high degree of specificity, because they are only ingested in the early larval phase, and because the exposure of humans and non-target insects to Cry proteins is extremely low.

Numerous animal safety studies conducted over the past 40 years have reported that Bt-derived insecticides are safe (summarized in Tables 2 and 3); however, other studies have raised a few potential toxicity concerns because these studies have demonstrated biological effects of Bt Cry proteins on mammals (discussed below and summarized in Table 4). The U.S. Environmental Protection Agency (EPA) supported the registration of Bt subspecies because these subspecies have failed to show any significant adverse effects with increases in body weight, upon clinical observation or upon necropsy (McClintock *et al*., 1995). Based on the current criteria to define the toxicity of compounds (Box 2), Bt products are considered safe; thus, they do not have to be chronically tested in more than one mammalian species as other chemical products do.

The initial toxicological studies of Bt bioinsecticides were performed with *Bacillus* species; however, the majority of studies have been performed with spores in rodents. Although mortality may occur during toxicological trials of other species in the *Bacillus* genus, no mortality occurred with *B. thuringiensis*; thus, Bt shows a high safety level compared with *B. anthracis* (the most virulent Bacillus to mammals). For example, the LD50 values of *B. anthracis*-derived spores were low, from 2.64 to 80 for intraperitoneal or subcutaneous injection compared with dosages used to assess the safety of Bt products one million- to one trillion-fold higher (10^{12} spores for mice) (Lamanna and Jones, 1963). This study clearly demonstrated the lethality only of higher doses of Bt spores and the lower toxicity in vegetative cells. However, the authors commented that the pathogenicity of Bt even in a low order of magnitude should forewarn us.

The safety of Bt spores was also studied in rats fed with 10^9 Bt spores daily for 730 days, without recording adverse reactions (Ignoffo, 1973). In contrast, Hernandez *et al.* (1998) reported for the first time that Bt spores could kill mice when applied at higher concentrations by the pulmonary route. These authors stated that 10^8 spores of the Bt serovars *konkukian*, *kurstaki*, *israelensis*, and *thompsoni* can cause 100%, 80%, 40% and 0% mortality, respectively, when the spores were administered intranasally (Hernandez *et al.,* 1998).

The toxicity level displayed by Bt spores did not affect their commercial use because mortality did not exceed the threshold dose specified by the regulatory agency (typically 10^6 cfu administered intravenously or intraperitoneally to mice) (Burges, 1981; Siegel, 2001).

Furthermore, acute exposure to a dose of 10^8 spores (tested in mice), which is considered equivalent to 10^{11} spores in man, has been argued to be highly unlikely to occur. Additionally, the conversion of these doses in mice to human equivalents on a cfu kg^{-1} basis to evaluate the possible damage effects is important and recommended. On this basis, for example, a dose of 1×10^6 cfu in a 20 g mouse is considered equivalent to a dose of 5×10^9 cfu in a 100-kg man (Siegel, 2001).

However, we recommend that this criteria be revised in view of the evidence provided in a few reports (discussed later in this review and in Table 4), showing that Bt-derived insecticides (bacterium, spores, protoxins, toxins or Bt crops) exhibited some physiological effects on mammals in studies performed using both *in vivo* and *in vitro* assays. For example, Lemos *et al.* (2013) tested three sub-lethal doses of the biological insecticide XenTari® WG (*B. thuringiensis* subsp. *aizawai*) administered orally and observed lesions in the kidneys, liver, lungs and reduced fertility in rats treated with the bioinsecticide at sub-lethal doses. However, no clinical signs of intoxication were reported, and neonates did not exhibit signs of malformations of the head, limbs, thorax or abdomen (Lemos *et al.,* 2013). However, notably, the material tested in this study was not a pure Bt toxin extract but a commercial formulation, which includes other potentially toxic ingredients such as inert agents including dispersants and surfactants. Indeed, pesticide formulations have been demonstrated to contain toxic

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Table 1. Specific toxins produced by genetically modified (GM) crops until 2014

Protein/crop	Developer	Product
Eggplant (*Solanum melongena*)		
Cry1Ac	Maharashtra Hybrid Seed Company (MAHYCO)	BARI Bt Begun-1, -2, -3 and -4
Potato (*Solanum tuberosum L*)		
Cry3A	Centre Bioengineering, Russian Academy of Sciences	Lugovskoi plus, Elizaveta plus, Atlantic NewLeaf™ potato, Atlantic NewLeaf™ potato, Atlantic NewLeaf™ potato
Cry3A	Monsanto Company	New Leaf™ Russet Burbank potato, Hi-Lite NewLeaf™ Y potato, Shepody NewLeaf™ Y potato, Superior NewLeaf™ potato
Rice (*Oryza sativa L*)		
CryAc, Cry1Ab	Huazhong Agricultural University (China)	BT Shanyou 63
CryAc, Cry1Ab	Huazhong Agricultural University (China)	Huahui-1
Soybean (*Glycine max L*)		
Cry1Ac	Monsanto Company	Intacta™ Roundup Ready™ 2 Pro
Tomato (*Lycopersicon esculentum*)		
Cry1Ac	Monsanto Company	---
Maize (*Zea mays L*)		
eCry3.1Ab	Syngenta	Agrisure® Duracade™
eCry3.1Ab, mCry3A, Cry1Ab, Cry1Fa2	Syngenta	Agrisure® Duracade™ 5122 Agrisure® Duracade™ 5222 Agrisure™ GT/CB/LL
Cry1Ab	Syngenta	Bt10, Agrisure™ CB/LL, Agrisure® Viptera™ 3110, NaturGard KnockOut™, Maximizer™
Cry34Ab1, Cry35Ab1, mCry3A, Cry1Ab, Cry1Fa2	Syngenta	Agrisure® 3122
Cry1Ab (truncated)	Syngenta	Agrisure® Viptera™ 2100
mCry3A, Cry1Ab,	Syngenta	Agrisure® Viptera™ 3111, Agrisure® Viptera™ 4, Agrisure® Viptera™ 3100, Agrisure™ CB/LL/RW, Agrisure™ 3000GT, Agrisure™ RW, Agrisure™ GT/RW
Cry1Ab, Cry1Fa2	Syngenta	Agrisure™ Viptera 3220
Cry9C	Bayer CropScience	Starlink™ Maize
Cry34Ab1 Cry35Ab1	Dow AgroSciences LLC and DuPont	Herculex™ RW
Cry34Ab1, Cry35Ab1,	DuPont (Pioneer Hi-Bred International Inc.)	Herculex™ RW Roundup Ready™ 2
Cry34Ab1, Cry1Ab, mcry3A	DuPont	Optimum™ Intrasect Xtreme
Cry1Fa2, Cry34Ab1, Cry35Ab1	Dow AgroSciences LLC and DuPont	Herculex XTRA™ RR
Cry1Fa2, mCry3A		Optimum™ TRIsect
Cry1Fa2	Dow AgroSciences LLC and DuPont	Herculex™ I RR
Cry1Ab	Monsanto Company	Roundup Ready™ YieldGard™ maize, YieldGard™, MaizeGard™, YieldGard™ VT Triple, YieldGard™ CB + RR, Liberty Link™ Yieldgard™ Maize
Cry1Ac	Monsanto Company	Bt Xtra™ Maize

(Continues)

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Journal of Applied Toxicology

Table 1. (Continued)

Protein/crop	Developer	Product
Cry1Ab	Renessen LLC (Netherlands) and Monsanto Company	Mavera™ YieldGard™ Maize
Cry3Bb1	Monsanto Company	YieldGard™ Rootworm RW, MaxGard™
Cry3Bb1, Cry1Ab	Monsanto Company	YieldGard™ Plus YieldGard™ Plus with RR
Cry3Bb1	Monsanto Company	YieldGard™ VT™ Rootworm™ RR2, YieldGard™ RW + RR
Cry2Ab2, Cry1A.105	Monsanto Company	YieldGard™ VT Pro™
Cry1A.105, Cry2Ab2, Cry3Bb1	Monsanto Company	Genuity® VT Triple Pro™
Cry1A.105, Cry2Ab2,	Monsanto Company	Genuity® VT Double Pro™
Cry1A.105, Cry3Bb1, Cry34Ab1, Cry35Ab1, Cry2Ab2,Cry1Fa2	Monsanto Company	Genuity® SmartStax™
Cry2Ab2, Cry1Fa2, cry1A.105,	Monsanto Company	Power Core™
Cry1Fa2,	Monsanto Company	Herculex™ I, Herculex™ CB

International Service for the Acquisition of Agri-biotech Applications (ISAAA). GMO Approval Database for insect resistance 2015.

adjuvants that are sometimes more toxic than their active principles and that could be responsible for secondary toxic effects (Mesnage *et al.,* 2013a). Therefore, we recommend that toxicological examinations of these bioinsecticide formulations be included to compare the purified toxins to determine precisely whether the toxic effects observed were attributed to the Cry toxins. Moreover, such further studies should also include an analysis of biochemical parameters to determine whether the changes or abnormalities found in the rats imply a loss of function of these organs. Additionally, studies that are more complete are required to indicate precisely whether the abnormalities detected were presented in all rats or what percentage of animals were affected because the above-mentioned studies only presented representative tissue samples and because the data presented suggested that sublethal doses administered can provide chronic toxicity in humans (Lemos *et al.,* 2013).

Although Bt bacteria are considered unable to develop infections in mammals, some studies have reported the persistence of bacteria in the lungs of rats 21 days after intratracheal instillation (Tsai *et al.,* 1997) or in the lungs of mice 2.5 days after inhalation exposure (Siegel *et al.,* 1987). Interestingly, Bt serovars *kurstaki* and *israelensis* bacteria persisted in the spleens of mice 37 and 49 days, respectively, after intraperitoneal injection (Siegel, 2001). Hence, converting these doses to a human equivalent on a cfu kg^{-1} basis to evaluate possible hazard and to perform immunological and allergenic tests is essential.

The first toxicological study with Bt that was performed in humans (Fisher and Rosner, 1959) is described in Box 3 while the infection cases are described below. Only three reports of Bt infection in humans were published in the latter half of the 1990s; because two of the registered infected patients had blast injuries, it was suggested they may have been immunocompromised. An important issue regarding these case reports is that they did not involve serovars used in commercial products (Damgaard *et al.,* 1997).

The first infection case was reported to be caused by a non-motile Bt (serovar undeterminable) and was recovered from two Italians with deep burns. The authors speculated that the contaminated water used to wash their burns and the immunosuppressed conditions of the patients made them susceptible to infection (Damgaard *et al.,* 1997).

Hernandez *et al.* (1998) presented the second infection case report; Bt *serovar konkukian* was isolated from the leg abscesses of a French soldier wounded by a land mine blast. To determine whether this isolate was infectious only in immunosuppressed people, 10^5–10^7 cfu were injected subcutaneously into immune intact and immunosuppressed mice. After 48h Bt *serovar konkukian* was recovered only from the tissue samples from immunosuppressed mice (Hernandez *et al.,* 1998).

Helgason *et al.* (2000) reported the third case of human infection, but the authors considered *Bacillus cereus* and Bt the same species (Helgason *et al.,* 2000). Thus, according to the current systematic classification of the genus *Bacillus*, this article is considered inaccurate (Sneath, 1986). In conclusion, only two cases caused by Bt species have been reported, and the infection were enhanced by the immunosuppressed conditions of the patients in extremely rare cases.

In contrast, epidemiological studies have addressed the possibility of increased incidence of infection and food poisoning with the large-scale spraying of Bt *serovar kurstaki* (Btk). The first study was performed in 1990 in the United States (Green *et al.,* 1990) in a sprayed area (with a population of approximately 80,000 people in 1985 and 40,000 people in 1986) in which cultures were obtained from humans during spraying periods. Bacteria were identified by microscopic examination; however, this methodology is not considered conclusive, as a high proportion of bacteria were misidentified with this technique. Fifty-five of 95 *Bacillus* isolates were identified as Btk, and 52 of these isolates were assessed to be probable contaminants whereas the three remaining Btk isolates could have been the cause of the patients' diseases.

The second was a microbiological and epidemiological surveillance study performed to monitor the health effects of Foray 48B, a spray pesticide that contains Bt var *kurstaki* (Btk). This study was conducted in a population of 1.4 million people living inside a spray zone in Canada (Noble *et al.,* 1992). The authors examined

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Table 2. Studies of *Bacillus thuringiensis* (Bt)-insecticides and their toxicity in mammals.

Bt-derivated	Cry gene	Study	Dose	Toxicity	Reference
kurstaki (Crymax)	Cry1Ac, Cry2A, Cry1C	Acute oral toxicity/ Patho-genicity (rat)	$>2.5–2.8 \times 10^8$ cfus per rat	No evidence of toxicity	(Carter and Liggett, 1994
kurstaki (Lepinox)	Cry1Aa, Cry1Ac, Cry3Ba	Acute oral toxicity/ Patho-genicity (rat)	$>1.19 \times 10^8$ cfus per rat	No evidence of toxicity	(Barbera, 1995)
kurstaki (Raven)	Cry1Ac, Cry3Aa, Cry3Ba	Acute oral toxicity/ Patho-genicity (rat)	$>4 \times 10^8$ cfus per rat	No evidence of toxicity	(Carter *et al*., 1993)
kurstaki (Cutlass)	Cry1Aa, Cry1Ab, Cry1Ac, Cry2A, Cry2Ab	Acute oral toxicity/ Patho-genicity (rat)	$>10^8$ cfus ml^{-1}	No evidence of toxicity	(**& David, 1988)
tenebrionis (San Diego)	Cry3Aa	Acute oral toxicity	>5050 mg kg^{-1}	No evidence of toxicity	(*EPA, 1991)
kurstaki (Dipel)	Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa	Acute oral (rat)	$>4.7 \times 10^{11}$ spores kg^{-1}	No evidence of toxicity	(*EPA, 1986)
kurstaki (Dipel)	Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa	13-week Oral gavage (rat)	$>1.3 \times 10^9$ spores kg^{-1}	No evidence of toxicity	(McClintock *et al*., 1995)
kurstaki (Dipel)	Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa	13-week Oral feed (rat)	>8400 mg kg^{-1} day^{-1}	No evidence of toxicity	(McClintock *et al*., 1995)
kurstaki (Dipel)	Cry1Aa, Cry1Ab, Cry1Ac, Cry2A	2-year chronic feed (rat)	8400 mg kg^{-1} day^{-1}	Statistically significantly decreased body weight gain in females from week 10 to week 104 (not considered related to Cry proteins); no infectivity/pathogenicity was found.	(McClintock *et al*., 1995)
kurstaki	Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa	Oral (Human)	1000 mg per adult or 1×10^{10} spores daily for 3 days	No toxicity/infectivity; all blood cultures were negative; 5 of 10 patients showed viable Bt microbes in stool samples 30 days post-feeding.	(*EPA, 1986) (McClintock *et al*., 1995)
berliner	Cry1Ab, Cry1B	5-day Oral exposure (Human)	1000 mg per adult or 3×10^9 spores in capsules daily for 5 days	All subjects remained well during the course of the experiment (5 weeks) and all laboratory findings were negative (subjects were evaluated before treatment, after =the 5-day treatment period, and 4 to 5 weeks posttreatment).	(Fisher and Rosner, 1959)
israelensis (Teknar)	Cry4A, Cry4B, Cry10A, Cry11A, Cyt1Aa	Acute oral toxicity/ infec-tivity (rat)	$>1.2 \times 10^{11}$ spores per kg	No evidence of toxicity	(McClintock *et al*., 1995)
israelensis (h-14)	Cry4A, Cry4B, Cry10A, Cry11A, Cyt1Aa	13-week Oral feed (rat)	>4000 mg kg^{-1} day^{-1}	No evidence of toxicity	(McClintock *et al*., 1995)

Modification of Siegel (2001) and Betz *et al*. (2000) & Unpublished study prepared for Ecogen, Inc. EPA MRID No.409511-02.

the records of more than 26 000 telephone calls reporting health-related problems and 3500 admissions to hospital emergency departments and closely monitored 120 workers with occupational exposure to Btk spray. Also, the study examined 429 bacterial cultures that had been referred in from 10 participating laboratories. Moreover, these authors examined samples of air and food to determine general and occupational concentrations of Btk. Almost two-thirds of the occupationally exposed spray workers developed some symptoms compared to one-third of the individuals in the control group who were not exposed. Many of the complaints included transient irritative effects such as eye, nose, and throat irritation; dry skin; a headache and chapped lips. Nearly all tested workers exposed to higher concentrations for several shifts (5–20) retained Btk for at least 5–6 days, and most were culture positive for 14–30 days. In contrast, 13 workers of 96 who were swabbed after an interval of 30 to 63 days as last Btk spray exposure yielded Btk from nose swabs, and 72% of them reported symptoms of sinus or throat irritation. However, no evidence of an increase in people with asthma or respiratory diseases or complaints of eye, nose or throat was found in people living inside the

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Table 3. Toxicity studies conducted with purified Cry toxins used test the biosafety of *Bacillus thuringiensis* (Bt) crops

Cry Toxin	Study	Results (no-effect levels) **mg kg^{-1} day^{-1}**	Toxicity	Dietary exposure	Reference
Cry1Ab	Acute oral toxicity (mouse)	>4000	No evidence of toxicity	>22 000 000 (corn)	(*EPA, 1996)
Cry1Ab	Acute oral oxicity (mouse)	>3280	No evidence of toxicity	>3 000 000 000 (corn)	(*EPA, 1995)
Cry1Ab	28-day mouse drinking water study	>0.45 via Drinking water	No evidence of toxicity, no evidence of immunological responses	>20 000 (tomato)	(Noteborn *et al.,* 1994)
Cry1Ab	31-day rabbit drinking water study	>0.06 via drinking water	No evidence of toxicity	>2600 (tomato)	(Noteborn *et al.,* 1994)
Cry1Ac	Acute oral toxicity (mouse)	>4200	No evidence of toxicity	>22 000 000 (cottonseed oil) >16 000 000 (tomato)	(*EPA, 1995)
Cry1Ac	Acute oral toxicity (mouse)	>5000	No evidence of toxicity	>560 000 000 (corn)	(Spencer *et al.*, 1996)
Cry3A	Acute oral toxicity (mouse)	>5220	No evidence of toxicity	>652 500 (potato)	(*EPA, 1995b)

Modified of Betz *et al.* (2000).

spray zone compared with those living outside the spray zone, nor were the symptoms more common in people who were found to be culture positive for Btk. The results of the physicians' office surveillance showed that the nose was easily able to trap Btk suspended in air. Of the 1140 patients examined, 128 (11.2%) of the nose specimens cultured were identified as Btk, with 58% from individuals living within the spray zone and 39.1% from outside the spray zone. However, the difference in the culture positivity rate among the zones was not statistically significant.

The laboratory surveillance programme showed that many people were exposed to Btk during a spraying period, as the bacterium was easily recovered from a broad range of body sites. Indeed, from 429 bacterial isolates submitted for analysis, 75.8% of isolates were characterized as Btk. Despite this finding, when critical specimens including blood, body fluids and tissues were examined by considering both the culture result and the patient information, these authors were unable to find a single case that fitted the study criteria where Btk was a pathogen-causing infection.

The third study was performed in May and June 1999 in Victoria, British Columbia, Canada, during aerial spraying. This study included (i) asthmatic children exposed to Btk (Pearce *et al.,* 2002), (ii) patients with infections from which Btk HD1-like bacteria were isolated and (iii) measurement of the distribution and incidence of Btk HD1-like isolates. These analyses were performed in the environment and a human population (Teschke *et al.,* 2001; Valadares De Amorim *et al.,* 2001). This study was supported by *cry* gene-specific PCR, random amplified polymorphic DNA analysis and DNA hybridization to screen over 11 000 isolates of bacteria. One thousand and nine individuals (randomly selected) were interviewed pre- and post-spray utilizing a symptom survey and a health status survey tool called the Short Form 12 Health Status Profile (SF-12) provided by Medical Outcomes. No significant differences were found following the aerial spray for reported symptoms or physical health score changes, and a small improvement in the average mental health score post-spray was reported for the residents both inside and outside of the spray zone. These results suggested that exposure to Btk HD1 did not have acute health effects on the general population (Levin, 2005).

In contrast, only two epidemiological studies have reported potential allergenic effects after exposure to Bt insecticides. The first study presented evidence indicating that exposure to Bt sprays may lead to allergic skin sensitization as well as to the induction of specific IgE and IgG antibody responses. This health survey was conducted in the muck crops region of northern Ohio from June to October 1995 and examined 126 farm workers before and after exposure to a commercial Bt pesticide, which consisted solely of the Btk strain. The workers were divided into three groups: the high exposure group (direct exposure to Bt spraying) and two groups of medium and low exposure (not directly exposed to Bt spraying). The evaluations included questionnaires to assess symptoms associated with allergic syndromes, collection of nasal/mouth lavages, assessment of ventilatory function, and tests of skin reactions to common aeroallergens and to a variety of Bt spore and vegetative preparations, and collection of sera for assessing specific IgG and IgE antibody responses to spore and vegetative Bt extracts. The majority of nasal lavage cultures from exposed workers were identified as Bt-positive, positive skin tests to several spore extracts were primarily detected in exposed

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Table 4. Effects of *Bacillus thuringiensis* (Bt) derivates on mammals

Product/ Bt species	Mode of exposure	Effects	Reference
Bacillus thuringiensis var. *kurstaki*	Populations were exposed to Bt spray.	Hundreds of people complained of allergic reactions. Exposed farm workers also exhibited increased antibody levels.	(Green *et al.,* 1990)
Contaminated water/ Undetermined	Opportunistic infection	Infection in burns of immunosuppressed patients.	(Helgason *et al.,* 2000))
B. thuringiensis serovar konkukian	Opportunistic infection	Leg abscesses of French immunosuppressed soldiers wounded by a land mine blast	(Hernandez *et al.,* 1998)
XenTari®/*B. thuringiensis subsp. aizawai*	Orally	Kidney, liver, and lung lesions and reduced fertility were observed in rats.	(Lemos *et al.,* 2013)
B. thuringiensis kurstaki and *B. thuringiensis israelensis*	Seven human cell lines were exposed	Bti/Btk products generated nonspecific cytotoxicities involving loss of bioreduction, and rounding, blebbing and detachment of cells.	(Tayabali and Seligy, 2000)
Roundup Ready corn	Rats were fed for 13 week with 11% or 33% corn	Slight increases in weight were observed.	(Hammond *et al.,* 2004)
Bt corn	Rats fed with Bt corn	Changes in creatinine, total protein and globulin levels were reported.	(Kilic and Akay, 2008)
Bt corn MON 810, MON 863 (modified Cry1Ab in MON 810, modified Cry3Bb1 in MON 863)	Rats fed with corn	The toxic effects were primarily associated with the kidney and liver as well as in the heart, adrenal glands, spleen and haematopoietic system.	(de Vendomois *et al.,* 2009)
Cry1Ab (<10 ng/ml)	Rumen cells of cows	The authors noted that Cry1Ab caused a four-fold increase in the conductance of potassium.	(Stumpff *et al.,* 2007)
Cry1Ab and Cry1Ac Bt toxins (10 ppb to 100 ppm)	Human embryonic kidney cell line 293 was exposed	Cry1Ab caused cell death from 100 ppm (0.1 mg/mL) compared to Cry1Ac, which has not effect on this cell line.	(Mesnage *et al.,* 2013b)
Cry1Ab toxin	Bovine hepatocytes were exposed.	The authors concluded that Cry1Ab has slight acute toxicity.	(Shimada *et al.,* 2006)

workers particularly 1 to 4 months after exposure to Bt spray. Moreover, specific IgG and IgE antibodies to vegetative Bt extracts were detected in all groups of workers but occurred primarily in the high exposure group (Bernstein *et al.,* 1999).

A second study that suggests possible allergenic effects of Bt bioinsecticides was a longitudinal epidemiologic study performed in > 300 Danish greenhouse workers. This study focused on respiratory health, including the prevalence and incidence of common changes in respiratory health such as lung function, status and changes as well as bronchial hyper-responsiveness. This study found that many sera had detectable specific IgE antibodies to Bt (23–29%) and concluded that microbial biopesticides may confer a risk of IgE-mediated sensitization and allergenic effects (Doekes *et al.,* 2004).

In contrast, Tayabali and Seligy (2000) conducted an extensive *in vitro* testing of Bt products and their subfractions using seven human cell types. The Bti/Btk products generated nonspecific cytotoxicities involving a loss of bio-reduction; rounding, blebbing and detachment of cells; degradation of immunodetectable proteins, and cytolysis (Tayabali and Seligy, 2000).

In conclusion, further and more complete toxicological studies with spore-containing Bt products are needed because the reports are contradictory. While various reports have indicated that these products have a high safety record, a few studies have shown that high exposure to these Bt insecticides may lead to allergic reactions, at least in some cases, for some Bt species.

Toxicological studies of transgenic plants expressing Cry proteins

Since its first commercialization in 1996, worldwide cultivation of transgenic crops expressing Cry toxins from Bt has occurred; several studies have been conducted to determine the safety of transgenic plants expressing Cry proteins for human consumption.

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Journal of Applied Toxicology

Table 5. Immune responses triggered by Bt derivates in mammals

Product/specie	Administration route	Effects	Reference
Bt-insecticides/ (*serovar kurstaki*),	Workers who were exposed for 2 years	Significant elevation in antibody titers were presented.	(Laferriere *et al.,* 1987)
Bt-insecticides	Higher-exposure exposure workers	Specific IgE and IgG antibodies to vegetative cells were presented in all groups. The authors stated that exposure to Bt sprays might lead to allergic skin sensitization, induction of IgE and IgG antibodies, or both.	(Bernstein *et al.,* 1999; Siegel, 2001)
Recombinant Cry1Ac protoxin from *B. thuringiensis* HD73	Intragastric and intraperitoneal	Cry1Ac protoxin has adjuvant properties and enhanced the immune response against co-administered antigens such as hepatitis B surface antigen (HBsAg) and bovine serum albumin (BSA).	(Vazquez *et al.,* 1999)
Recombinant Cry1Ac protoxin from *B. thuringiensis* HD73	Intranasal	This protoxin induced IgM, IgG and IgA antibodies in serum, vaginal and tracheo-bronchial washes and in the fluids of the large and small intestine and increased the antibody titer against pneumococcal polysaccharides.	(Moreno-Fierros *et al.,* 2000; Moreno-Fierros *et al.,* 2003)
Recombinant Cry1Ac protoxin from *B. thuringiensis* HD73	Mucosal and systemic	Soluble Cry1Ac protoxin improves protection against *Brucella abortus* or three different mouse models of parasitic infections such as those caused by *Naegleria fowleri*, *Taenia crassiceps*, *Plasmodium chabaudi* and *Plasmodium berghei ANKA AS*.	(Ibarra-Moreno *et al.,* 2014; Legorreta-Herrera *et al.,* 2010; Rojas-Hernandez *et al.,* 2004)
Recombinant Cry1Ac protoxin from *B. thuringiensis* HD73	Intranasal	Cry1Ac protoxin induced significant antibody cell responses and increased the activation of T cells and cytokine production in nasal-associated lymphoid tissue (NALT) and lymphocytes of nasal passages.	(Rodriguez-Monroy and Moreno-Fierros, 2010)
Recombinant Cry1Ac protoxin from *B. thuringiensis* HD73	*In vivo* and *in vitro* Cry1Ac protoxin	Activate murine macrophages of primary cultures or cell lines. This protoxin also induced the overproduction of proinflammatory cytokines through MAP kinase pathways such as MEK and p38.	(Moreno-Fierros *et al.,* 2013).
Recombinant Cry1Aa, Cry1Ab and Cry1Ac toxins from *B. thuringiensis* HD73	Systemic and nasal	These toxins induced IgG and IgA antibodies in different mucosal compartments.	(Guerrero *et al.,* 2004)
Recombinant Cry1Aa wild type and mutant toxins from *B. thuringiensis* HD73	Intranasal route	These toxins generated cellular immune responses in mice and promoted interferon-gamma production.	(Guerrero *et al.,* 2007).
Cry1Ab toxin	Intraperitoneal	This toxin is immunogenic and may induce a mixed Th1/Th2 immune response, with intense production of anti-Cry1Ab IgG1 and IgG2a antibodies.	(Adel-Patient *et al.,* 2011).

Acute toxicity testing, along with determining digestive fate *in vitro*, is considered sufficient to test health risks from dietary exposure to Cry proteins expressed in plants (Sjoblad *et al.,* 1992), and no adverse effects have been observed in acute oral mammalian toxicity studies conducted in mice with Cry1, Cry2 and Cry3 ranging up to 5220 mg kg^{-1} body weight. However, as discussed below, Cry proteins produced by Bt plants are considered safe based on limited evaluations performed in experimental animals. It has been assumed that the Cry proteins are digested in the human gastrointestinal fluid. However, although Cry1Ab protoxin and toxin were extensively degraded at pH 1.2 and with a high pepsin-to-protein ratio (usual condition of the simulated gastric fluid of the tests performed for assessment allergenicity), Cry1Ab proteins were stable and conserved its immunoreactivity using a physiologically more relevant digestion model (pH 2.5, pepsin-to-protein ratio 1:20 w/w) (Guimaraes *et al.,* 2010). Moreover, it has been argued that Bt plants have no risk of toxicity because the exposure levels are thousands to millions of times higher than the

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Box 1. Cry toxin receptors on insect cells

APN

Belongs to the aminopeptidase protein family. In the lepidopteran larval midgut, this protein digests proteins derived from the insect's diet and works in cooperation with endopeptidases and carboxypeptidases (Wang *et al.,* 2005). APN belongs to the zinc binding metalloprotease superfamily that cleaves neutral amino acids from the N terminus of polypeptides and that has been extensively studied as Cry toxin receptors. Cry toxins can bind differentially to APN; for example, Cry1Ac toxin binds trough domain III to *N*-acetylgalactosamine (GalNAc) moieties present in APN receptor. The primary binding domains on Cry1Ac were determined and involved the residues 509QNR511, N506 and Y513 (Burton *et al.,* 1999). In contrast, in Cry1Aa, the domains involved in binding to *Bombix mori* APN (Nakanishi *et al.,* 2002) are located at domain III in two amino acid regions: 508STRVN513 and 582VFTLSAHV589 (Atsumi *et al.,* 2005).

Cadherin-like receptor

Calcium-dependent adhesion proteins, also named cadherins, are transmembrane proteins that play important roles in cell adhesion, migration, cytoskeletal organization, and morphogenesis (Gumbiner, 1996; Angst *et al.,* 2001). Classic cadherins are located primarily within adherens; lepidopteran cadherin-like proteins have been identified on the apical membrane of midgut columnar epithelial cells and are exposed and interact with Cry toxins (Bravo *et al.,* 1992; Braun and Keddie, 1997; Atsumi *et al.*, 2005; Midboe *et al.,* 2003; Aimanova *et al.,* 2006). The primary regions that interact with Cry1A toxins on CADR are three residues: 865NITIHITDTNN875 is located in repeat 7 and interacts with Cry1Ab at loop 2, loops a-8, loop 2 interacts with residues 1331IPLPASILTVTV1342 located in repeat 11 in the CADR receptor (Gomez *et al.,* 2002, 2003), and residues 865NITIHITDTNN875 located in repeat 7 on CADR binds to Cry1A.

ALP

GPI-anchored alkaline phosphatases have been characterized as Cry1A binding proteins in *H. virescens* and *M. sexta* and are included in lipid rafts in cell membranes. Lipid rafts are enriched in glycosphingolipids, cholesterol and GPI-anchored proteins and are proposed to be involved in signal transduction and in sorting and trafficking of plasma membrane proteins (Munro, 2003). The interaction of pore-forming toxins with lipid rafts could result in additional cellular events, including toxin internalization, signal transduction and cellular response; however, studies regarding this subject are very limited.

Other binding proteins

Cry1Ac also binds to V-ATPase subunit A and actin, indicating that the mode of action of Cry toxins may involve binding of the toxin with other components of the midgut cells; however, their roles in the mechanism of action remain to be analyzed (McNall and Adang, 2003).

Box 2. Criteria to define toxicity

To determine the toxicity of any compound, first, we need to define toxicity as the degree to which a substance or agent is able to generate damage on an exposed organism. Toxicity is often estimated as the quantity of the agent that needs to be administered to obtain an observable effect (lethal or sublethal) in a given proportion (usually 50%) of the test population (van Frankenhuyzen, 2009). To determine the safety of transgenic proteins, the best strategy that has been developed consists of two parts. The first part is to determine the potential hazard (Tier I), which involves the assessment of the action mode and biological function of the protein. Tier I is divided in some tests such as 1) history of safe use, 2) bioinformatics analysis, 3) mode of action, 4) *in vitro* digestibility, 5) stability, and 6) expression level and dietary intake. The next step is conducted when the results from Tier I are not sufficient to determine the safety and includes the hazard characterization (Tier II). This last part involves 1) acute toxicology, 2) repeated dose toxicology study and 3) hypothesis-based evaluations. All safety studies of Bt products have included intraperitoneal or intravenous injection in laboratory animals and the subsequent quantitative recovery of Bt from their tissues (Adlersberg *et al.,* 1969). Mice have been widely used to test acute toxicity of the transgenic proteins. Acute toxicological studies are performed with a single exposure to high concentrations of the protein, feeding mice with 2–5 g kg^{-1} body weight one time by oral route; next, fed animals are observed daily for 14 days for body weight changes and clinical signs of adverse effects. When the time period is ended, the obvious pathological changes to major organs are determined (Delaney *et al.,* 2008).

potential dietary exposure (Betz *et al.,* 2000); however, these estimations also need to be revised, using accurate methods to quantify the protein content in GM plants and considering the variations in the expression levels of Cry proteins in plants (Székács and Darvas, 2012).

In addition, Bt crops are considered to have not shown any adverse effect to mammals based on the absence of mortality, records of normal body weight, and lack of abnormalities detected after gross pathology examinations performed in necropsy of animals fed with these crops (Table 3). However, notably, conflicts of interest exist in the case of the review of Betz *et al.* (2000), as it was published on behalf of the Monsanto Company. Another important point to consider in relation to the review of Betz *et al.* (2000) is that only some of the existing experimental works assaying the toxicity of GM crops were cited. Moreover, some of the cited experimental works supporting the safety of GM crops were also published on behalf of the Monsanto Company. Furthermore, the EPA re-registration eligibility decision form used to approve the use of microbial pesticides based on Bt stated that the safety of these pesticides is supported by a historical toxicological database reviewed by McClintock *et al.* (1995) and by the safety studies performed with spores and complete bacillus but not with

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Box 3. First safety record of *Bacillus thuringiensis* (Bt) bioinsecticides

To determine the safety record of Bt-derivate insecticides, many studies were performed (McClintock *et al.,* 1995); human volunteers were tested in some of the early studies. In 1959, Fisher and Rosner reported the effects of thuricide insecticide (15% of *Bacillus thuringiensis* var. *kurstaki*, also including spores and cellular debris), which was ingested by 18 humans (1000 mg daily for 5 days) or inhaled by five of these subjects (100 mg of biopesticide daily) for 5 days. At the end of the fifth day, the subjects where submitted to physical and laboratory examinations, and the tests were repeated 4 or 5 weeks after the last ingestion/inhalation. In addition to these tests, individuals who inhaled insecticide were also subjected to X-ray examinations in the same time range. Medical examinations included a detailed record of height and weight, temperature, blood pressure, breathing and pulse rate (Fisher and Rosner, 1959). Assessments of genitourinary, gastrointestinal, cardiorespiratory and nervous system were also included. Laboratory tests included routine analysis of urine, urobilinogen determination, complete blood counts, sedimentation rate, blood urea nitrogen, glucose and bilirubin. The results were compared with the study tests took before the administration.

The authors reported that the individuals remained well during the experiment. Although the tests were accomplished to determine the general organ and system functions, more complexes examinations are needed to discard any possible adverse effects of Bt products such as the degree of activation of the immune system, the potential allergenicity of thuricide, or the possible chronic effects, which remained unclear. The earliest long term toxicology test (7 months) was performed with eight Biofarm employees in different parts of the manufacture and control of thuricide production, two of the employees had been exposed greater than any other workers (total exposure, 251 h to all phases of production and control); however, they were in excellent health and did not show evidence of chronic or acute damage of any kind from exposure to Bt. Nevertheless, the tests performed were too general to exclude possible additional effects such as immunological reactions.

the GM insect-protected plants. Furthermore, most toxicological evaluations tested purified toxins instead active soluble toxins produced by the plants. This point is important to mention because GM plants express genetically modified Cry toxins, not the native Bt proteins. For example, maize varieties in the MON 810 variety group have been reported to produce a truncated form of the bacterial Cry1Ab protoxin because a single, preactivated Cry1Ab toxin of approximately 91-kDa molecular mass that is further proteolytically activated in the midgut of the insect larvae (Székács and Darvas, 2012). The results of the evaluation of native proteins are assumed to be comparable to those of the GM-proteins; however, as a consequence of modifications, their effects on mammals or their reactivity may be quite different. Moreover, indicating that the integration of Cry genes in the plant genome could cause a complex recombination generating new proteins that might be quite different compared to the native toxins (Rosati *et al.,* 2008) is important. Therefore, the debate continues regarding issues related to the persistence and adverse effects of Bt proteins on mammals and non-target organisms in part because the toxicological tests performed thus far are very superficial (Janer *et al.,* 2008).

Another irregularity with the regulatory rules applied to commercialize Bt products is that these rules do not require 3-month tests with three mammalian species, followed by evaluations in a mammalian species for 1 year and another test for 2 years, although these rules are required to approve chemical pesticides and drugs. In contrast, scarce data have been published regarding mid- or long-term toxicological studies with mammals.

The reports of long-term feeding with transgenic Bt corn have been conducted on rats or mice; in general, these reports have not shown significant toxicological effects. For example, no alterations in body weights or kidney or liver function were observed in rats after feeding for 90 days with a diet of Bt-corn expressing Cry1Ab protein (Schroder *et al.,* 2007). In another study where rats were fed for 13 weeks with 11% or 33% Roundup Ready corn, the unique change found was that male rats showed slight increases in weight (Hammond *et al.,* 2004).

Likewise, the histopathological analysis performed in male and female rats fed with transgenic Bt corn showed no apparent differences throughout two generations (Polat, 2005). Similarly, in the studies accomplished to test the safety of Bt crops where rats and mice were fed with GM soybean for 105 days showed no histopathological abnormalities in the mucosa of the small intestine (Teshima *et al.,* 2000).

Toxicological studies performed in cows from 2005 to 2007 in which 18 lactating dairy cows were fed with transgenic MON810 maize or with the near-isogenic counterpart (N = 18) revealed the absence of potential toxicological effects (Guertler *et al.,* 2012). The gene expression profile was investigated, and compared to near-isogenic feed, MON810 maize did not have any observed effect on major genes involved in apoptosis, inflammation and the cell cycle in the gastrointestinal tract and the liver of dairy cows. Likewise, when Buzoianu *et al.* (2012) evaluated the effects of feeding pigs with Bt maize MON810 during gestation and lactation, these authors did not observe data that could indicate inflammation in pigs fed for 143 days throughout gestation and lactation (Buzoianu *et al.,* 2012). Thus, these studies support the safety assessment of Bt maize.

In a further effort to exclude any adverse effects caused by the ingestion of transgenic crops, Tripathi *et al.* (2011) assessed the effect of transgenic crops on lambs fed with genetically modified Bt cotton seed expressing the Cry1Ac protein; however, no changes in haematology, blood biochemistry or histopathology were observed.

Adel-Patient *et al.* (2011) found that neither non-transgenic maize nor MON810 maize produced allergenic-related profiles or immune responses against maize. In that study, proteins were administered to mice at quantitatively equivalent amounts (Adel-Patient *et al.,* 2011).

Walsh *et al.* (2012) developed a method to evaluate the potential long-term toxicity of Cry1Ab (110 days) and the age-specific effects on pigs fed with genetically modified Bt maize. These authors found that the effects on the peripheral immune response and digestive fate did not show allergic- or inflammation-related immune responses. No evidence of antigen-specific antibody production, overproduction of inflammatory cytokines or changes in T cell populations was found (Walsh *et al.,* 2012).

The final study conducted to approve the MON863 maize by the European and American authorities was performed by (Hammond

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et al., 2006) under the responsibility of the Monsanto Company, and this GM maize was approved. This study was performed using 6-week-old rats separated into 10 groups of 20 males and 10 groups of 20 females. The tests included overall health, body weight gain, food consumption, clinical pathology parameters (haematology, blood chemistry, and urinalysis), organ weights, and gross and microscopic appearance of tissues. These parameters were comparable between the groups fed diets containing MON 863 and conventional corn varieties. These authors confirmed that MON863 maize is as safe and nutritious as the existing wild-type corn varieties.

However, some statistical-related issues regarding this MON863 study were raised by Seralini *et al.* (2007) when these authors reanalysed the raw data obtained by Hammond *et al.* (2006) with other statistical analyses. These authors concluded that the statistical methods used by Monsanto were not detailed enough to see disruptions in biochemical parameters and were not able to detect possible signs of pathology within only 14 weeks (Hammond *et al.,* 2006). The two primary organs of detoxification, the liver and kidney, apparently had been disturbed; however, this study strongly recommended a new assessment and longer exposure of mammals to these diets, with cautious clinical observations, before concluding that MON863 is safe to eat (Seralini *et al.,* 2007).

Likewise, potentially toxicological effects on rats were suggested by the statistical reanalysis of the raw data published by the Monsanto Company (Hammond *et al.,* 2006) performed by de Vendomois *et al.* (2009) in which the effects of MON 810 and MON 863 (containing modified Cry1Ab and modified Cry3Bb1, respectively) had been tested. The effects analysed consisted of 60 different biochemical parameters, which were evaluated in rats after 5 and 14 weeks of feeding. The reanalysis of the data indicated that the toxic effects were primarily associated with the kidney and liver as well as with the heart, adrenal glands, spleen and haematopoietic system (de Vendomois *et al.,* 2009). Moreover, because the toxicological evaluation of the study supported by the Monsanto Company was performed only in rats, this evaluation needs to be repeated preferably with more than one animal species. Also, the length of the feeding was for only three months; thus, only relatively acute and medium-term effects could be observed. Therefore, additional long-term (up to 2 years) animal feeding studies should be performed in at least three distinct species, and preferably, these studies should also be multi-generational to provide true scientifically valid data regarding the acute and chronic toxic effects of feeding with GM crops.

Despite, the aforementioned reports sustaining that any toxicity is associated with the consumption of Bt crops, some studies have demonstrated different effects caused by these Bt plants. For example, changes in creatinine, total protein and globulin levels were reported in a biochemical analysis performed in rats fed with Bt corn that has insect resistance for the most invasive corn borer (Kilic and Akay, 2008).

In a study in which the effect of MON810 maize was evaluated in the gut and peripheral immune response of mice fed under vulnerable conditions (weaning and old mice), some immunological alterations were recorded such as changes in lymphocyte subpopulations in the gut and peripheral sites in mice fed with MON810 maize compared to mice fed with control maize. MON810 maize induced changes including alterations in the percentages of B, CD4+ and CD8+ cells, including cells with the phenotypes $\gamma\delta$T and $\alpha\beta$T. Also mice fed for 30 or 90 days had increased levels of serum IL-6, IL-13, IL-12p70 cytokines and MIP-1 (Finamore *et al.,* 2008).

Kroghsbo *et al.* (2008) studied the possible immunogenic and allergenic effects of consuming Bt rice expressing Cry1Ab. In that study, Wistar rats were fed for 28 days with control rice, transgenic Bt rice or transgenic Bt rice spiked with 0.1% purified recombinant Bt toxin (Cry1Ab). Only spiked transgenic Bt rice was found to be immunogenic, as it showed augmented IgG1 levels in comparison with the other groups. Moreover, the observed anti-Cry1Ab antibody response was induced after both inhalation (control groups) and inhalation/ingestion in groups fed recombinant protein alone or together with transgenic rice, but no adverse effects of the Cry1Ab protein were found (Kroghsbo *et al.,* 2008).

Although a large variation in measurements of Cry toxin concentrations in plant material, in general, has been reported, the expression levels recorded in transgenic plants are assumed to be low. For example, Cry1Ab in MON810 maize was estimated to be 0.0013% of the protein content (Adel-Patient *et al.,* 2011). Based on these estimations, it has been argued the amount of Cry1Ab that could be ingested by consuming MON810 maize does not represent any risk. Given that a 60-kg person would have to eat 18 461 kg day^{-1} corn to achieve the highest fed dose tested in mice (4 g kg^{-1}), which is considered non-lethal (Table 3). However, if we consider that an estimated dose of Cry1Ac of approximately 0.3–2 mg kg^{-1} is needed to achieve a biological effect (for example, an immunostimulating effect with Cry1Ac via the intranasal route in mice can be elicited with doses of 5–50 μg per 25-g mice (Guerrero *et al.,* 2004; Moreno-Fierros *et al.,* 2013). Therefore, the estimated intake of corn to achieve these doses would be highly reduced, and a 60-kg individual may present an immunostimulating effect by eating from 1.38–9.23 kg of corn. Moreover, in other transgenic plants such as broccoli, the expression levels of Cry proteins are known to achieve higher levels (0.4% of total soluble protein); therefore, even lower amounts of these GM plants would be required to be ingested to achieve these doses (Cao *et al.,* 1999).

Finally, an additional point that should be considered for the precise toxicological evaluation of transgenic plants expressing Cry toxins is precisely related to the expression levels of these Cry toxins, which are highly variable. Indeed, the Cry protein content in transgenic insect-resistant maize may vary between tissues within plants and between plants growing under different environmental conditions. For example, the expression levels of the Cry1Ab toxin were demonstrated to be 9.6–17.2, 2.3–5.3 and 1.4 mg g^{-1} in the leaves, roots and stalk of *MON 810* maize DK-440 BTY, also showing a seasonal fluctuation of Cry toxin expression (Székács *et al.,* 2010).

Also, fertilization may also increase Cry1Ab toxin production due to the higher biomass of maize varieties due to N-fertilization (Bruns and Abel, 2003). Moreover, Cry1Ab toxin production varies among Bt maize varieties produced by different genetic events (Székács *et al.,* 2010; Székács and Darvas, 2012). Furthermore, in a recent report, large variations in transgene expression and Cry protein content were caused by the genetic background of the maize variety and by environmental conditions, indicating that the concentration of Cry protein is even more difficult to predict under stressful conditions (Trtikova *et al.,* 2015). Therefore, toxicological evaluations of GMOs should be performed with different tissues from plants cultured under different environmental conditions and the expression levels of Cry proteins should be rigorously monitored regularly in the distinct GMO cultures to be able to detect opportunely significant changes in the Cry protein content.

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In vivo and *in vitro* toxicological studies with Cry purified toxins in mammalian cells

Thus far, most studies support that Cry toxins are not toxic to mammalian cells. However, these toxins do not appear to be innocuous. Fundamental features of the Cry proteins remain unclear, and their effects on mammalian cells have not yet been completely studied. From our point of view, the application of *in vitro* cell culture systems particularly for the preliminary screening of GM foods might offer many advantages such as to attain sufficient results at low costs and at high speed and to decrease the number of animal used, as these advantages of *in vitro* tests have been highlighted for screening the toxicity of new compounds (Luber-Narod *et al.,* 2001).

One of the most used toxins in transgenic plants and bioinsecticides is Cry1Ab. Various studies have reported that this toxin has low oral acute toxicity in mice (LD_{50} was >5000 mg kg^{-1} body weight). However, Bt toxins have been detected in mammals, suggesting these toxins are being accumulated; the 50% lethal concentrations (LC_{50}) of Bt toxins range from 10 to 520 ppb (Rani and Balaraman, 1996; Ito *et al.,* 2004; Nagamatsu *et al.,* 2010).

Supporting the safety of Cry toxins, acute toxicity studies conducted in mice fed with Cry1Ab protein in GM crops at a dose $>$ 4000 mg and Cry1Ac $>$ 4200 mg administered by the oral route indicated that Cry1Ab and Cry1Ac proteins have no toxic effects on animal models (Xu *et al.,* 2009).

However, evidence indicating that these toxins are not innocuous also exists. For example, Stumpff *et al.* (2007) examined the effects of Cry1Ab in the rumen cells of cows ($<$10 ng ml^{-1}) at 37 °C and found no significant effects at a concentration of 100 ng ml^{-1}. However, interestingly, the authors noted that Cry1Ab caused a four-fold increase in the conductance of potassium (Stumpff *et al.,* 2007). Similarly, the protoxin Cry1Ac also has been shown to exert some physiological effects on mammalian cells, as it binds *in situ* to the intestinal epithelium of mice and induces transient hyperpolarization of the mucosal tissue (V´azquez-Padrón *et al.,* 2000). Also, as mentioned later, our group has demonstrated that the protoxin Cry1Ac can induce murine macrophage activation both *in vitro* and *in vivo* (Moreno-Fierros *et al.,* 2013).

Bondzio *et al.* (2008) reported that sheep rumen epithelial cells were an appropriate *in vitro* model to determine the possible toxic potential of toxin Cry1Ab, no toxicity was observed in this model in short- and long-term experiments even at the higher non-physiological concentrations tested. However, later in 2013, this research group performed a study in porcine intestinal cell cultures (IPEC-J2) in which the effects of valinomycin, a cytotoxic agent, were compared with those of the Cry1Ab toxin. Although no toxicity was observed after 24 h of Cry1Ab treatment, up-regulation of a heat shock protein (Hsp70) was noted. Given that the doses used were very low (0.5 μg ml^{-1} for Cry1Ab and 1 μg ml^{-1} for Cry1Ac) (Bondzio *et al.,* 2013) and some additional physiological effects were shown, higher doses should be tested.

In another study, Mesnage *et al.* (2013b) tested the effects of Cry1Ab and Cry1Ac Bt toxins (10 ppb to 100 ppm) on the human embryonic kidney cell line 293; Cry1Ab caused cell death at 100 ppm (0.1 mg ml^{-1}) compared with Cry1Ac, which had no effect on this cell line. These authors found that the combination of both toxins reduced caspase 3/7 activation, which is induced by Roundup insecticide, and could delay apoptosis. Based on their results, these authors argued that modified Bt toxins are not inert on non-target human cells and that they can present combined side-effects (Mesnage *et al.,* 2013b).

Shimada *et al.* (2006) did not observe significant changes in the secretion of albumin or the morphology of bovine hepatocytes incubated with Cry1Ab toxin; nevertheless, the authors concluded that Cry1Ab has slight acute toxicity on bovine hepatocytes (Shimada *et al.,* 2006). However, Teixeira Correa *et al.* (2012) reported that no toxic effect was observed for the trypsin-activated Cry toxins Cry4Aa and Cry11A in breast cancer cells (MCF-7) when tested at 20 mg/mL (Teixeira-Correa *et al.,* 2012).

In contrast, toxicological studies have also been performed with chimeric proteins expressed in GM plants. Xu *et al.* (2009) performed both *in vitro* and *in vivo* animal studies, and their results supported the safety of Cry1Ab/Ac proteins, showing that these proteins do not possess the characteristics associated with allergens, as they can be rapidly degraded in gastric and intestinal fluids. Additionally, when mice were fed with 5 g Cry1Ab/Ac protein/kg body weight for 14 days, no signs of morbidity or mortality caused by Cry1Ab/Ac were observed. Data obtained from blood biochemistry and haematological studies at day 15, when the study was terminated, did not show any statistically significant difference between male and female mice fed with Cry1Ab/Ac proteins compared with the controls groups. In general, organ weights, gross necropsy and histopathology did not have any Cry-related alterations (Xu *et al.,* 2009).

Immune responses triggered by Bt derivates

Commercial Bt products are a mixture of vegetative cells, spores, spores undergoing germination and cell debris from vegetative cells (Siegel *et al.,* 1987), which may trigger an immune response in mammals (Table 5). Laferriere *et al.* (1987) reported a significant elevation in antibody titers in workers who were exposed to Bt insecticides (*serovar kurstaki)* for 2 years; however, antibodies disappeared 1 year after exposure (Laferriere *et al.,* 1987).

Bernstein *et al.* (1999) reported that specific IgG and IgE antibodies were present in higher-exposure than low-exposure workers. Specific IgE and IgG antibodies to vegetative cells were present in all groups. The authors stated that exposure to Bt sprays might lead to allergic skin sensitization, induction of IgE and IgG antibodies, or both. However, no increase in the incidence of asthma or other occupationally related clinical diseases was observed in higher exposure workers (Bernstein *et al.,* 1999).

We have extensively described above that Bt toxins, Bt crops and Bt derivates are safe but non-innocuous for vertebrates. One of the major changes induced by the exposure to these products is reflected in the immunological system, which is specialized to recognize strange or harmful antigens. Several of the reported physiological effects in mammals imply the activation of the immune system as we list here. (1) When populations were exposed to Bt spray, hundreds of people complained of allergic reactions; exposed farm workers also exhibited increased antibody responses levels (Samples and Buettner, 1983; Green *et al.,* 1990). (2) Bt toxin induced a significant immune response in male rats fed with MON 863 Bt corn including increased basophil, lymphocyte and total white cell counts (Burns, 2002). (3) Our investigation group has demonstrated that the Cry1Ac protoxin is a potent mucosal and systemic immunogen and adjuvant (Vazquez-Padron *et al.,* 1999; Moreno-Fierros *et al.,* 2003). The high immunogenicity of the Cry1Ac protoxin was demonstrated by its capacity to induce significant specific antibody responses in serum and mucosal secretions recovered from the small and large intestine, broncho-alveolar and vaginal lavages of mice after immunization by every tested route: intraperitoneal, intragastric, intranasal, rectal

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(Vazquez *et al.,* 1999; Moreno-Fierros *et al.,* 2000) and vaginal (Moreno-Fierros *et al.,* 2002). The adjuvant effects of Cry1Ac protoxin have been evaluated regarding the specific antibody responses attained at both mucosal and systemic levels, to co-administered antigens of different nature (proteins such as surface antigen of hepatitis B (Vazquez *et al.,* 1999), HIV peptides (Esquivel-Perez and Moreno-Fierros, 2005) and pneumococcal polysaccharides (Moreno-Fierros *et al.,* 2003). Moreover, the mucosal adjuvant effect elicited by the Cry1Ac protoxin can be as potent as that elicited by Cholera toxin (Moreno-Fierros *et al.,* 2003), although the effects depend on the administration route and the antigen used. Also, the Cry1Ac protoxin can function as a vaccine carrier when conjugated to 6B pneumococcal polysaccharide, as it enhanced the systemic and mucosal-specific antibody responses (Moreno-Fierros *et al.,* 2003).

Furthermore, various studies from our group sustain the potential utility of the Cry1Ac protoxin as a promising protective adjuvant capable of improving vaccine efficacy. Indeed, soluble Cry1Ac protoxin co-administered either as adjuvant or even alone confers protection against three distinct mouse models of parasitic infections such as those caused by *Naegleria fowleri*, *Taenia crassiceps*, *Plasmodium chabaudi* and *Plasmodium berghei* ANKA AS (Rojas-Hernandez *et al.,* 2004; Legorreta-Herrera *et al.,* 2010; Ibarra-Moreno *et al.,* 2014). Moreover, the Cry1Ac protoxin increased the immunoprotection conferred by the RB51 vaccine in mice challenged with the virulent strain *Brucella* abortus 2308 (Gonzalez-Gonzalez *et al.,* 2015).

The immunopotentiating properties of the Cry1Ac protoxin may be related to its ability to activate lymphocytes and antigen-presenting cells. Because when this protein is intranasally administered to mice, it is able to induce an increased proportion of activated T and B lymphocytes and an increased proportion of T lymphocytes producing cytokines and significant specific IgA and IgG antibody cell responses in nasal lymphocytes isolated from both nasal-associated lymphoid tissue (NALT) and nasal passages (NP) (Rodriguez-Monroy and Moreno-Fierros, 2010). In contrast, when Cry1Ac was co-administered intranasally as adjuvant with *Naegleria fowleri* lysates, only this treatment induced metaplasia in the olfactory epithelium and increased IgA secretion compared to immunization with lysates alone, effects that may be related to the increased protection conferred by this treatment; however, this result deserves further analysis (Jarillo-Luna *et al.,* 2008).

In addition, we have demonstrated that the Cry1Ac protoxin is able to activate murine macrophages of both primary cultures or cell lines *in vivo* and *in vitro*, inducing the expression of co-stimulatory molecules such as CD80 and CD86 and the overproduction of proinflammatory cytokines including MCP-1, IL-6 and TNF-α. In macrophages, the activation mechanism of the Cry1Ac protoxin appears to be mediated through MAP kinase pathways such as MEK and p38 (Moreno-Fierros *et al.,* 2013).

Moreover, we also found that Cry1Aa, Cry1Ab and Cry1Ac toxins administered by systemic and nasal routes in mice were highly immunogenic and were able to induce IgG and IgA antibodies in different mucosal compartments (Guerrero *et al.,* 2004). Furthermore, both wild-type and mutant Cry1A toxins (in which an eight amino acid hydrophobic motif in a-helix 7 of wild-type Cry1A toxins was exchanged for a diphtheria toxin epitope) were able to generate cellular immune responses and to increase cytokine production, particularly interferon-gamma production, in mice immunized by the intranasal route (Guerrero *et al.,* 2007).

Using a BALB/c mouse model, Adel-Patient *et al.* (2011) confirmed the immunogenicity of the Cry1Ab toxin, which may induce a mixed Th1/Th2 immune responses when it is administered intraperitoneally (using doses of 1 or 100 μg administered at days 1 and 15). Moreover, a specific antibody response to Cry1Ab was characterized by the intense production of IgG1 and IgG2a antibodies (Adel-Patient *et al.,* 2011). Likewise, Andreassen *et al.* (2015) confirmed the previously reported immunogenicity of Cry1Ab by the intranasal route (Guerrero *et al.,* 2007).

Based on the specific anti-Cry1Ab IgG1 and IgE production recorded, this protein may have inherent immunogenicity and allergenicity (Andreassen *et al.,* 2015).

Further directions and concluding remarks

The majority of laboratory studies that were performed to test the infectivity and toxicity of Bt commercial products have indicated that these products are safe; nevertheless, such studies are not enough proof that these products are innocuous to mammalian cells or vertebrate organisms. Some of the studies conducted regarding the effects of Bt-derived insecticides have demonstrated the capacity of these toxins to activate the immune system and to increase the humoral antibody responses and have suggested that these toxins could produce allergic responses.

Producers of GM crops such as Bt crops assume that Cry proteins expressed in transgenic plants will not be allergenic, because they will be rapidly degraded in gastric fluid, as occurs with pure Cry proteins treated with simulated gastric fluid, at pH 1.2 and with high pepsin to protein ratio. However, these tests should be assessed both *in vivo* and *in vitro* using physiologically relevant digestion models. Furthermore, because Cry proteins are not similar to any described allergenic protein and because human toxicological studies performed with spores or complete bacillus did not show toxicity, Bt crops are assumed to lack toxicity or adverse effects. Importantly, in the cooking process of some of these crops such as maize and rice, toxins could be modified or inactivated; however, this possibility must be tested experimentally not only theoretically. Other crops such as eggplant (recently marketed) and tomato do not require cooking processes for consumption; therefore, the ingestion of the protein would be direct.

We believe that expanding the parameters for evaluating the toxicity of Cry proteins, not only the general aspects such as mortality, loss of weight and vital signs but also more comprehensive exams of the systems, organs, tissues and cells, is necessary. The effects on the gastrointestinal tract, the immune system or the genitourinary tract have yet to be determined; although the EPA has decided that acute toxicological evaluation is sufficient to evaluate the risk and safety of new Bt products that are manufactured.

Our group has demonstrated the immunogenicity of the Cry1Ac protein in mice, its adjuvant effect and its ability to activate murine macrophages *in vivo* and *in vitro*; however, the immunogenicity of these proteins and their possible risks in humans after short- and long-term exposure must be determined. Evaluation of the risks of Cry proteins in other systems such as the respiratory and nervous systems is also needed. The toxicity definition must include the adverse effects caused by these toxins not only in the short term; therefore, subchronic and chronic studies in humans should be performed, and the immunotoxicological features of these toxins should be determined.

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Conflict of interest

The studies, opinions and analysis in this review were objective and supported by several previously conducted studies. The authors have no conflicts of interest or financial conflicts regarding the management of this information. We only based this review on the overall scope of this topic and its potential benefit to science.

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* Regulatory reports

**Implicit Conflicts of interests

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