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Commentary

Assessment of potential adjuvanticity of Cry proteins

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ABSTRACT

Genetically modified (GM) crops have achieved success in the marketplace and their benefits extend beyond the overall increase in harvest yields to include lowered use of insecticides and decreased carbon dioxide emissions. The most widely grown GM crops contain gene/s for targeted insect protection, herbicide tolerance, or both. Plant expression of *Bacillus thuringiensis* (*Bt*) crystal (Cry) insecticidal proteins have been the primary way to impart insect resistance in GM crops. Although deemed safe by regulatory agencies globally, previous studies have been the basis for discussions around the potential immuno-adjuvant effects of Cry proteins. These studies had limitations in study design. The studies used animal models with extremely high doses of Cry proteins, which when given using the *ig* route were co-administered with an adjuvant. Although the presumption exists that Cry proteins may have immunostimulatory activity and therefore an adjuvanticity risk, the evidence shows that Cry proteins are expressed at very low levels in GM crops and are unlikely to function as adjuvants. This conclusion is based on critical review of the published literature on the effects of immunomodulation by Cry proteins, the history of safe use of Cry proteins in foods, safety of the *Bt* donor organisms, and pre-market weight-of-evidence-based safety assessments for GM crops.

1. Introduction

Microbial products derived from various strains of the common soil bacterium *Bacillus thuringiensis* (*Bt*) have been used as insecticides since the 1930s (Ibrahim et al., 2010). No harmful or adverse effects have been demonstrated after occupational exposure to *Bt* products, and no adverse effects have been reported in the consumer population exposed to these products in the form of spray residues on conventional or organic crops (WHO, 1999). The crystal proteins that confer insecticidal properties to *Bt* sprays are

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Abbreviations

APC	antigen-presenting cell
BSA	bovine serum albumin
Bt	*Bacillus thuringiensis*
Cry	Crystal
DON	deoxynivalenol
EFSA	European Food Safety Authority
Fb_1	fumonisin B_1
GM	genetically modified
GMO	genetically modified organism
HbsAg	hepatitis B surface antigen
HOSU	history of safe use
ig	intragastric
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
in	intranasal
ip	intraperitoneal
LPS	lipopolysaccharide
Maalox	magnesium-aluminum hydroxide suspension
PPB	parts per billion
PPM	parts per million
US	United States

highly specific to a subset of immature insects and have been widely used in genetically modified (GM) crops to confer insect protection. The mammalian and environmental safety of Cry proteins have been well studied and they are as safe in *Bt* spray products used in conventional and organic farming and forestry as they are in *Bt*-based GM crops (Federici and Siegel, 2007). Extensive uses of Cry protein-containing products and safety studies have provided robust evidence of vertebrate safety. Interestingly, this safety profile has prompted some preliminary investigation of the potential for Cry proteins, like Cry1Ac, to act as medically safe vaccine adjuvants in animals (Jarillo-Luna et al., 2008; Moreno-Fierros et al., 2003; Rojas-Hernandez et al., 2004; Vazquez et al., 1999). Although the experimental design has been questioned, these studies concluded that Cry proteins can act as adjuvants.

Adjuvants are commonly used to enhance the efficacy of vaccines by inducing a stronger and more durable immune response (Brunner et al., 2010). Typically, Cry protein studies investigating adjuvanticity used classic vaccination strategies, which focus on immunizing by systemic exposure to both the antigen and adjuvant. This contrasts with the oral exposure scenarios of *Bt*-sprayed crops or novel proteins in GM foods. Nevertheless, the interpretation of Cry protein studies investigating adjuvanticity of Cry proteins has led to postulation of adjuvanticity for Cry proteins in humans and, by extension, possible enhancement of immune responses to dietary components (EFSA, 2009). In this review, we will discuss the use of Cry proteins in agriculture, the safety of Cry proteins, and the literature concerning potential adjuvanticity of the Cry proteins.

2. History of safe use of the *Bt* microorganism and Cry proteins

Bacillus thuringiensis is a common bacterium present in soils, on grains, and in environmental habitats including the phylloplane and water (Martin and Travers, 1989). Not only is *Bt* found in the environment, it is also found in many animals including voles, deer, rodents, and insectivorous mammals, as well as in processed food products, such as pasta, bread and other foods that contain flour (OECD, 2007). Numerous *Bt* strains produce insecticidal Cry proteins or inclusion bodies that are effective in controlling certain species of insect pests (Aronson and Shai, 2001). *Bt* biopesticides have been adopted for use in commercial agriculture, forestry, and mosquito control (OECD, 2007). Because of their robust insecticidal activity, preparations of whole *Bt* organisms have been used by farmers since the 1920s, being sold commercially in France (as Sporine) as early as 1938, and registered as an insecticide in the United States (US) beginning in 1961 (Ibrahim et al., 2010). As of 2011, more than 100 microbial *Bt* products have been registered to provide effective control of insect pests (US-EPA, 2011). Despite the years of occupational exposure from aerial application of *Bt* microbial sprays to forests and agricultural crops, only one scientific report describes potential respiratory or dermal sensitization to *Bt* pesticides (Bernstein et al., 1999). The report describes skin prick reactivity and antibody responses to *Bt* spray formulations in farm workers, but these responses did not correlate well with exposure and do not appear to be directed against the spore or delta endotoxin component of the products. The lack of toxicity of *Bt* spores has been established by other studies: one clearly demonstrated that human acute oral exposure to live spores (1 g/day for 5 days) resulted in no toxic effects, nor did inhalation of 100 mg of Btk powder daily for 5 days (Fisher and Rosner, 1959), Another showed a lack of correlation between the presence of *Bt* in feces collected from greenhouse workers after exposure and any gastrointestinal ill effects (Jensen et al., 2002). Based on these and additional studies, the World Health Organization and others have concluded that *Bt* sprays containing Cry proteins have demonstrated no adverse health effects in workers who apply them, nor in the general public in the areas where the sprays are applied (WHO, 1999) (Federici and Siegel, 2007). Because of their demonstrated safety, *Bt* formulations are often sprayed on crops such as broccoli, tomatoes, cucumbers, cauliflower, and lettuce plants for insect control just prior to harvest (Frederiksen et al., 2006). These crops are eaten raw and typically with only minimal washing, meaning humans have been safely consuming *Bt* spores and Cry proteins (Federici and Siegel, 2007). Thus, in over 75 years of commercial use as biopesticides, *Bt* insecticidal proteins have been used and consumed safely with no reported adverse effects on human health or the environment (McClintock et al., 1995; Siegel, 2001).

The first GM crop generated by engineering the plant genome to express an insecticidal Cry protein was approved in the US in 1995. The safety of the plant-expressed Cry proteins in GM crops was supported by the evidence from decades of safe use of these same proteins in microbial sprays. Modification of plants to express specific insecticidal proteins is a safe and highly effective method for insect control for which safety has been confirmed through mammalian and environmental safety assessments (Betz et al., 2000). In addition, GM crops expressing Cry proteins have been grown since 1995 with no documented reports of adverse health effects (AMA, 2012; Betz et al., 2000; Key et al., 2008; Toxicology, 2003; US-EPA, 2005).

Cry proteins expressed in GM crops have been evaluated in accordance with internationally recognized guidelines for assessing potential allergenicity and toxicity (Codex, 2009; FAO/WHO, 2001). The results indicate that Cry proteins show no relevant amino acid

sequence similarity with known human allergens or toxins, are heat labile, and have negligible concentrations in food (typically ppm levels in the raw unprocessed commodity). These are all characteristics that have supported the safe introduction of Cry proteins from both an allergy and toxicity perspective (Betz et al., 2000). Immunotoxicological evaluation of GM maize expressing Cry1Ah by a 30-day feeding study showed no significant differences in histology of immune organs, spleen/thymus weights or immune function parameters in mice fed with GM maize compared to those fed with conventional maize (Song et al., 2014). There are no reports showing that *Bt* causes clinical allergy despite its long history of use and consumption (US-EPA, 2001) and there are no other immune-related clinical data that indicate a disease state related to *Bt* exposure. Postmarket studies evaluating antibody responses in appropriate populations consuming various Cry proteins expressed in transgenic maize and soybean were conducted. No significant levels of specific IgE antibodies to various Cry proteins were detected in the sera of susceptible patients, suggesting that Cry proteins are not likely to cause allergic reactions (Batista et al., 2005; Kim et al., 2009; Mathur et al., 2015; Nakajima et al., 2007). Adjuvanticity is an immune-related phenomenon that has never been observed for Cry proteins in humans. Adjuvanticity has been raised as a potential safety concern based on some laboratory studies designed to evaluate the potential of Cry proteins (almost exclusively Cry1Ac) to initiate immune responses in animals. The European Food Safety Authority (EFSA) concluded that consumption of Cry proteins via food/feed derived from *Bt* maize does not present a safety concern for the health of humans or animals (EFSA, 2009).

3. Definition, mechanism of action, and various types of adjuvants

Adjuvants are immunostimulatory compounds that can increase and/or modulate the immunogenicity of an available antigen, leading to stronger and longer-lasting immune responses (Brunner et al., 2010). Adjuvanticity is the immunomodulatory ability exerted by adjuvants, which are typically added to vaccine preparations to enhance immunizations. This enhancement is achieved by modifying the microenvironment in which the antigen is presented, influencing the development of antibody-as well as cell-mediated immune reactions. Adjuvants can be classified in a number of different ways based on their source, mode of action, or physicochemical properties. In general, adjuvants operate by one or more of the following mechanisms: 1) increasing delivery of antigens through depot formation or carrier activities, 2) triggering the secretion of cytokines from antigen-presenting cells (APCs) or stimulatory molecules from other cell types, and 3) enhancement of antigen uptake and presentation (Brunner et al., 2010). A number of adjuvants also act through combined mechanisms; complete Freund's adjuvant, for example, contains a depot-generating emulsion along with heat-killed mycobacteria to directly activate APCs (Billiau and Matthys, 2001).

Many naturally-derived materials have been used as immunization adjuvants, and these are often found in the diet. Plant-derived saponins like the *Quillaja* saponins derived from the soap bark tree are potent adjuvants in animal vaccines (Fleck et al., 2006; Rajput et al., 2007). Such saponins are present in commonly consumed foods like quinoa as well as various legumes (e.g. soy), and thus they have a history of safe use (Estrada et al., 1998; Oda et al., 2003; Verza et al., 2012). Many food plants contain lectins, which are glycoproteins with carbohydrate binding properties. While few of these have been tested for outright adjuvanticity, many of them stimulate the immune system, upregulating expression of antigen presentation machinery and cytokines, and stimulating T-cell proliferation (Cordain et al., 2000). Chitin, a fungal cell wall component found in mushrooms and yeast, as well as its food supplement derivative chitosan, are known mucosal adjuvants (Asahi-Ozaki et al., 2006; Da Silva et al., 2010; Svindland et al., 2013) and yet they are also commonly present in foods. Taurine, an amino acid consumed in the diet and a common ingredient in energy drinks, is effective as an oral adjuvant in mice and humans (European Commission, 1999; Hayes and Trautwein, 1994; Ishizaka et al., 1990; Kuriyama et al., 1988). Other adjuvants encountered through the diet or dietary supplements include lithium (Ishizaka et al., 1990; Lieb, 1987; Shenkman et al., 1980), sodium fluoride (Butler et al., 1990; Hoshi et al., 1999; Kuriyama et al., 1988), and various Japanese and Chinese herbal medicine ingredients (Rajput et al., 2007; Song and Hu, 2009; Taguchi et al., 2012; Wang et al., 2005). Often touted as 'immune-boosting', these foods and supplements have a history of safe use.

Conserved microbial structures, such as lipopolysaccharide (LPS), peptidoglycan, and flagellin, are capable of immunostimulation and hence are recognized as adjuvants (Cuadros et al., 2004; Halassy et al., 2003; Wang and Singh, 2011). Although these adjuvants are pathogen-associated structures, pathogenicity is not a result of these microbial structures being adjuvants. In the diet, these come from safe bacteria in cultured and fermented foods (Cho et al., 2014; Meydani and Ha, 2000; Taverniti and Guglielmetti, 2012). Additionally, the commensal organisms that safely colonize the mucosa provide a substantial repository of these and other microbial components with immunostimulatory capacity (Licciardi and Tang, 2011).

4. Review of studies assessing potential adjuvanticity of Cry proteins

Vaccine strategies include administration of adjuvants to improve mucosal immunity (Elson and Dertzbaugh, 1994). Cholera toxin and heat-labile enterotoxin are known to have strong adjuvant effects when administered with poor mucosal immunogens but are not suitable for use in vaccines due to toxicity (Clements et al., 1988; Lycke and Holmgren, 1986). Cry proteins are known to be resistant to proteolysis, soluble and stable in highly alkaline pH and are innocuous to vertebrates (Hofte and Whiteley, 1989). These beneficial properties make Cry proteins an interesting alternative for the development of carriers of relevant epitopes in vaccines. So, adjuvanticity of Cry proteins has been investigated to explore their potential as vaccine adjuvants (Esquivel-Perez and Moreno-Fierros, 2005; Jarillo-Luna et al., 2008; Legorreta-Herrera et al., 2010; Moreno-Fierros et al., 2003; Rojas-Hernandez et al., 2004; Roman Calderon et al., 2007; Vazquez et al., 1999; Verdin-Teran et al., 2009). In these studies, mice were exposed to microgram amounts of soluble or crystallized forms of Cry1Ac protoxin either intragastrically (*ig*), intranasally (*in*) or intraperitoneally (*ip*). The *in* and *ip* routes of exposure are very specific and powerful systemic routes of exposure, respectively. In contrast, putative adjuvant effects in foods would occur through the oral route. For the purpose of discriminating whether Cry proteins possess adjuvanticity under the expected conditions of dietary exposure, only the study using the *ig* route of administration is considered relevant (Vazquez et al., 1999). In this study, co-administration of Cry1Ac and hepatitis B surface antigen (HbsAg) or bovine serum albumin (BSA) was observed to enhance serum anti-HbsAg and anti-BSA IgM, IgG, and IgA antibody responses. Cry1Ac administered by the *ig* route in this study included co-administration with 100 μl of magnesium-aluminum hydroxide suspension (Maalox®); an antacid containing aluminum hydroxide. It is important to note that this vehicle protects proteins from digestion, prolonging their interaction with the intestinal immune system. Even more

importantly, the use of aluminum hydroxide was not appropriate since it is an established adjuvant, known to potentiate immune responses via direct stimulation of immune cells (Mannhalter et al., 1985; Rimaniol et al., 2004). Thus, this assessment of adjuvanticity was confounded by the presence of an adjuvant in the vehicle. Additionally, potential contaminants derived from bacterial production of these proteins were not measured; LPS is a typical contaminant that can be present and co-purified when preparing recombinant protein preparations. LPS and peptidoglycan can function as oral adjuvants (Inagawa et al., 2011; Murakami et al., 1994; Ogawa et al., 1986; Sun et al., 2014) and in some cases these components can have a synergistic impact in combination with aluminum compounds to stimulate immune responses (De Gregorio et al., 2008; Eisenbarth et al., 2008). Thus methodological flaws in this study do not allow a valid conclusion that Cry1Ac can act as an adjuvant, de novo and in isolation.

The potential adjuvanticity of Cry1Ab (a protein with 86% amino acid homology to Cry1Ac) has been investigated in a mouse model designed for peanut allergy (Guimaraes et al., 2008). In contrast to the adjuvant cholera toxin, no augmentation of peanut-specific antibody by purified Cry1Ab (including peanut-specific IgE) was observed in this model. Similarly, there was no adjuvant effect of Cry1Ab on cytokine responses generated in antigen-recall experiments. Upon respiratory challenge with peanut, levels of leukotrienes, some cytokines, and lung eosinophils were increased in the group treated with Cry1Ab and peanut. However, statistical significance was lacking for each of these measurements when compared with either the naïve or peanut alone control groups. The doses of Cry proteins in these studies were 10 and 100 μg, which is equivalent to 5000 μg/kg on a body weight basis. For an average adult human, this would be 350 mg of protein. Expression levels of Cry proteins in seed and grain varies, ranging from 0.3 μg/g for Cry1Ab in Yieldgard® Cornborer maize up to 115 μg/g for Cry1F in Herculex 1® Insect Protection maize (fresh weight), for example (Hammond and Cockburn, 2007). Cry1Ac expression in DBT418 maize kernels was reported to range from 0.36 to 0.43 μg/g (dry weight). If this maize were consumed at the 97.5th percentile consumption rate (UK), the exposure to Cry1Ac protein would be approximately 0.77 μg/kg, or 53.9 μg for an average adult, thousands of times less than the experimental doses used in the animal study.

In other examples of studies examining potential immune-related effects of Cry proteins, the focus has been on a more relevant study design whereby oral exposure to the GM grain in the animals' diet is employed. A thorough examination of the literature for these types of publications revealed few studies, all of which were Cry1Ab-related. A recent study designed to assess potential adjuvanticity of Cry1Ab expressed in MON810 maize fed mice GM and non-GM maize as 33% of the diet and showed that immune responses to dairy proteins in the diet were not augmented by the presence of Cry1Ab protein in the GM grain (Reiner et al., 2014). This study also demonstrated that inclusion of Cry1Ab in the diet did not affect the initiation or severity of ovalbumin-induced asthma and allergic inflammation (including lung eosinophils), nor did it enhance ovalbumin-specific antibody production. Similarly, Adel-Patient et al. (2011) demonstrated equivalent antibody responses directed toward maize proteins in mice after *ig* administration of extracts of MON810 maize vs. non-GM maize with cholera toxin, suggesting that the presence of Cry1Ab did not influence immune responsiveness.

Kroghsbo et al. (2008) reported that no immune-related effects were associated with Cry1Ab in a study of rats fed a diet incorporating either 60% Cry1Ab-expressing transgenic rice or rice spiked with purified protein for 28 or 90 days. As a control, kidney bean lectin was included as a treatment. Unlike Cry1Ab, the lectin induced increased IgA production and increased mesenteric lymph node weights; thus, this model represents one of the better controlled study designs because of the addition of this control treatment. A confirmatory study in rats fed a *Bt* rice-containing diet or a Cry1Ab-spiked diet had no effect on the antibody response to immunization with sheep red blood cells, indicating a lack of Cry1Ab adjuvanticity (Kroghsbo et al., 2008).

Some of these studies highlight a contamination concern similar to that noted for purified Cry proteins preparations. In a better understood model of immunology, mice fed MON810 maize grain or the conventional counterpart as 50% of the diet for 30 or 90 days (Finamore et al., 2008) had altered lymphocyte profiles in the intestine, blood, and spleen. These changes were observed along with altered cytokine levels. However, in this study the mycotoxins fumonisin B_1 (FB_1) and deoxynivalenol (DON) were reported to exceed maximum allowable levels in the two maize hybrids and were present in opposite ratios. Both FB_1 and DON are known to be immunomodulatory, with suppression or stimulation being observed depending on the model, dose, and duration of exposure (Martinova, 1997; Pestka, 2010; Sobrova et al., 2010; WHO, 2000). For FB_1, levels were 2450 and 1350 μg/kg in the control and GM maize, respectively. For DON, concentrations were 650 μg/kg in the control maize and 1300 μg/kg in the GM maize. Thus both mycotoxins could have contributed to immune skewing in either group. A higher response to a *Salmonella* vaccine in the absence of other immune alterations was observed in the group consuming grains produced by Cry1Ab-expressing maize in a three-year feeding study of ewes and their offspring (Bt176 maize, 100–600g/day) (Trabalza-Marinucci et al., 2008). However, mycotoxin content of the grain was not assessed, and since certain mycotoxins are known to bolster immune responses (Sobrova et al., 2010), this observation could not necessarily be solely attributed to the exposure to Cry1Ab.

Overall, studies done to assess the immunomodulatory potential of Cry1Ac have major flaws in the design that preclude valid interpretation. For Cry1Ab, studies were better executed and controlled, but evidence of reproducibility is lacking as there are no studies employing the same model with the same protocol.

Protein adjuvants, by their nature, elicit immune responses to themselves (Borsutzky et al., 2003; Güereña-Burgueño et al., 2002; Petrovsky and Aguilar, 2004; Vajdy et al., 2004). For this reason, immunogenicity, or the ability to provoke an immune response, is an integral part of a discussion on adjuvanticity. Although immunogenicity should not be taken as evidence of adjuvanticity (or allergenic potential), a lack of immunogenicity is a general indicator that the protein is not capable of stimulating the immune system and consequently would not act as an adjuvant. Any protein can be made to be immunogenic, particularly by the systemic route, which is the basis of general antibody production in animals for use in diagnostics. Examining Cry protein reactivity after co-exposure with adjuvants in animals, systemically, is unlikely to be a model that translates to indicating risk for human food exposure. Moreover, since Cry proteins are consumed orally, peripheral routes of Cry protein immunogenicity should not be considered and only mucosal routes of immunogenicity are relevant for further examination.

The single feeding study purported to detect Cry1Ab-specific antibodies detected them in both treated and control groups, with exposure being attributed to inhalation of Cry1Ab-containing dust (Kroghsbo et al., 2008). As this was an uncontrolled exposure in a study that also included an immunostimulatory lectin, immune responses to Cry1Ab cannot be attributed to exposure via the diet. The other feeding studies measuring Cry-specific antibodies after exposure to Cry-expressing GM crops detected no Cry1Ab-specific antibody response (Adel-Patient et al., 2011; Buzoianu et al.,

2012; Walsh et al., 2011). Additionally, no Cry1Ab-specific antibody responses were generated after *ig* administration of MON810 maize extracts with cholera toxin (Adel-Patient et al., 2011).

In studies aimed at characterizing the immunogenicity of the Cry1Ac protein, mice were exposed to microgram amounts of soluble or crystallized forms of Cry1Ac by the *ig* route (Vazquez-Padron et al., 1999, 2000). As in the study by Vasquez et al. previously described (Vazquez et al., 1999), the proteins were co-administered with 100 μl of magnesium-aluminum hydroxide suspension (Maalox®); an antacid containing aluminum hydroxide which can act as an adjuvant (Mannhalter et al., 1985; Rimaniol et al., 2004). The authors concluded that Cry1Ac was a strong immunogen based on antibody responses following three exposures. However, the effects of the vehicle cannot be discounted, as it is not only an adjuvant but also an antacid with potential to enhance immunogenicity by prolonging interaction with the immune system. Additionally, sufficient characterization of the purified Cry1Ac is lacking. Information on the purity of the protein preparations with specific attention to known immunostimulatory contaminants such as LPS is critical in supporting an accurate interpretation of study data. Because the soluble and crystallized forms of the Cry1Ac protein used in these experiments were isolated from two different bacterial sources, the elicited antibody responses may in fact have resulted from contaminants in the Cry1Ac protein preparations. Given the flaws in test material characterization and delivery, these data provide little information with respect to Cry1Ac's immunogenicity in and of itself, under precise oral exposure conditions where co-exposure to immune-modulating agents is completely controlled for in the experiment. For Cry1Ab, a protein with 86% amino acid homology to Cry1Ac, no anti-Cry1Ab antibodies were detected in mice after *ig* administration of 100 μg of purified protein assessed for LPS (Adel-Patient et al., 2011), even though such responses were detected in mice after the same dose of Cry1Ac by this route by another investigator (Vazquez-Padron et al., 2000). In contrasting their results with those from the Cry1Ac study, Adel-Patient et al. (2011) acknowledged potential LPS contamination of Cry1Ac as an influential factor.

Introduced Cry proteins are expressed at low levels in the grain/seed of the commercialized GM crops. Cry1Ab expression level in MON 810 GM maize event was detected to be 0.83 ± 0.15 ppm in the grain (Szekacs et al., 2010). Similarly, Cry1F expression levels in Herculex 1® GM maize were between 71 and 115 ppm (Mendelsohn et al., 2003). Mean expression levels of Cry1Ac in DBT418 maize kernels ranged from 36 to 42.8 ng/g dry weight (FSANZ 2002 safety assessment of DBT418 maize). According to the WHO/GEMS database, the consumption rate of maize in human is 4.98 g/kg/day; therefore 42.8 ng/g Cry1Ac corresponds to around 213 ng/kg of Cry1Ac for humans. In contrast, proteins with known immunomodulatory activity (lectins) are present in the food supply at much higher concentrations; cereals contain 13–53 ppm wheat germ agglutinin, for example (Ortega-Barria et al., 1994). The estimated dietary intake of total lectins ranges up to 2.85 mg/kg (2,850,000 ng/kg) per day (Watzl et al., 2001). Thus, it is reasonable to assume that the low level presence of introduced Cry proteins in food pose minimal risk of exposure to cause immune or adverse responses to themselves under the conditions of exposure from *Bt* crops. This is another indicator that Cry proteins in food are unlikely to exert adjuvanticity.

In summary, study design is highlighted as a critical aspect in discerning the potential use of animal models to identify adjuvant effects from Cry protein exposure. The main concern appears to be the co-exposure to unwanted toxicants or known immune-modulating molecules such as LPS or mycotoxins when preparing purified Cry protein or test diets. In those studies that best control for unwanted contaminants, and also use positive, immune-modulating controls, the results clearly support the hypothesis that Cry proteins likely have low or no immune effects through the oral route. This places a premium on investigators' characterization of these test materials (grain or purified protein) prior to implementing exposure protocols, whether in ewes or in mice.

5. Discussion

Cry proteins, through their use as biopesticides and expression in GM crops, have a long history of safe use (since the time *Bt* sprays were first used in agriculture) and are widely consumed via foodstuffs derived from conventional, organic, and transgenic agriculture. Thus, there is an established history of safe use through the oral route of exposure. Some studies of the potential adjuvanticity of Cry proteins have prompted questions about whether ingestion of Cry proteins could result in immunomodulation in humans. These studies evaluated potential Cry protein adjuvanticity within laboratory animal vaccine models; models not appropriate for assessing oral exposure. To monitor immune-related endpoints that could presumably indicate the presence of Cry1Ac adjuvanticity, some studies employed concentrated doses of *Escherichia coli*-derived protein preparations of unproven purity with uncharacterized excipients and contaminants. Additionally, the Cry proteins were typically co-administered with an antacid known for being an adjuvant and inhibiting pepsin activity, thereby enhancing immunogenicity and confounding adjuvanticity assessment. To date, no study has replicated the putative adjuvant-related effects observed. Taken together, the available data are not sufficient to conclude that the Cry1Ac protein or any other Cry protein can act as an adjuvant by the oral route or to refute the long history of safe exposure to these proteins and conflicting data showing no immunostimulatory response. Proper characterization of starting materials is critical for any scientifically rigorous study and particularly for analysis of immunomodulation where contaminants are likely to influence outcomes (Franken et al., 2000; Raybould et al., 2013). Also, more than one contaminant could be present with unaccounted and potentially synergistic interaction among them and/or the test agent. To properly address and support the assessment that there is no safety concern for Cry protein adjuvanticity, a well-defined and well-controlled study design that tests Cry protein adjuvanticity using well-characterized starting materials and optimised exposure scenarios would be required.

As risk assessment is a function of hazard and exposure, even in the unlikely event of there being an identified adjuvant property of Cry proteins, exposure is negligible for these types of proteins. Processing of food crops subjects proteins to chemical, thermal, and mechanical stresses, eliminating their functional activity (Hammond and Jez, 2011), which argues for Cry proteins losing native protein structure and inherently reducing exposure to the protein as it exists within the unprocessed grain. Moreover, given the oral route of exposure, novel proteins in GM products are assessed for their propensity to enzymatically break down when exposed to the gut and the associated immune system; this is a standing key feature of current allergy safety assessments. The known lability of Cry proteins to gastric digestion in combination with low dietary exposure levels (i.e., ppm or ppb) supports a conclusion that it is highly unlikely that Cry proteins, as expressed in GM crops, have any potential to act as an adjuvant. Since there is no evidence that the Cry proteins have any sequence similarity to known mitogens or lectins and the host plants (expressing the Cry proteins) are not commonly allergenic, there is no reason to consider the test for adjuvanticity of the Cry protein or the GM plant. Also, known protein adjuvants tend to be toxins (e.g. cholera toxin). No toxic effects for Cry proteins are observed at extremely

high doses (e.g. 4000 mg/kg) (Hammond and Cockburn, 2008). The adjuvant hazard and oral exposure to Cry proteins in food is extremely low, and thus, the overall risk for Cry proteins to act as adjuvants is negligible. This is consistent with the assessment by the EFSA Genetically Modified Organisms (GMO) panel whereby they concluded that there is not a safety concern for the health of humans or animals that consume food/feed derived from GM maize (EFSA, 2009) containing Cry proteins.

Transparency document

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