Human and Ecological Risk Assessment, 12: 856–870, 2006

ISSN: 1080-7039 print / 1549-7680 online
DOI: 10.1080/10807030600826805

RESEARCH ARTICLES

Assessing Risk of Unintended Antigen Occurrence in Food: A Case Instance for Maize-Expressed LT-B

Jeffrey D. Wolt,[1] Kan Wang,[2] and Robert K. D. Peterson[3]
[1]Biosafety Institute for Genetically Modified Agricultural Products, Iowa State University, Ames, Iowa, USA; [2]Center for Plant Transformation, Iowa State University, Ames, Iowa, USA; [3]Agricultural and Biological Risk Assessment, Department of Entomology, Montana State University, Bozeman, Montana, USA

ABSTRACT

Plant-based antigen production represents an innovative strategy for low cost vaccine production and delivery. Successfully advancing plant-made antigen production in open field systems requires understanding of confinement integrity and consequences of inadvertent occurrence in the food supply. The food safety implications of confinement loss and inadvertent antigen occurrence in the food supply can be effectively addressed using quantitative exposure assessment along with knowledge of properties of specific antigens. We report here a food safety risk assessment for the maize-expressed heat-labile enterotoxin subunit B of *Escherichia coli* (LT-B). In addition to dietary exposure assessment, food safety considerations for maize-expressed LT-B included assessment of allergenic potential, levels and sites of transgenic protein expression, history of use, post-translational glycosylation, protein processing and digestive stability, mammalian functionality and toxicity, and compositional characteristics of the transformed plant. As shown for LT-B, inadvertent occurrence in the food supply of a plant-produced antigen constitutes a minimal human health concern principally because of limited exposure potential.

Key Words: *Escherichia coli*, enterotoxin subunit B, vaccine, transgenic, confinement.

INTRODUCTION

Genetically engineered plants producing protein antigens have rapidly emerged as a novel paradigm for pharmaceutical production with large promise and equally large uncertainty regarding public understanding of their safety and regulation. The state of regulatory science for assessing the risks associated with inadvertent

Received 7 November 2005; revised manuscript accepted 14 December 2005.
Address correspondence to Jeff Wolt, Biosafety Institute for Genetically Modified Agricultural Products, Iowa State University, Ames, Iowa 50011, USA. E-mail: jdwolt@iastate.edu

occurrence of transgenically expressed protein antigens and other biologics in foods is evolving. An unanswered issue for open field production of plant transgenic biologics is that they are not intended as food, yet they may be grown in food crops. Adopting a science-based risk assessment paradigm for production of plant-made biologics, which is focused on quantitative exposure assessment and product attributes, provides a means to address both public and regulatory expectations regarding all aspects of safety for these products (including indirect as well as direct routes of exposure).

Together exposure and hazard describe the risk as the probability of an unwanted consequence for confinement loss for a protein antigen produced in an open field environment. The exposure itself is a function of the level of expression of the protein antigen in the plant and the degree of confinement integrity for this expressed agent. Hazard (intrinsic toxicity of the plant-expressed agent) and expression (concentration of the agent in the transformed plant) are product-specific attributes. Confinement integrity, however, can be generally described and predicted relative to the specific cropping system and management practices that are used (Christensen *et al.* 2005). Confinement integrity has been described quantitatively as a first step in understanding food or feed adulteration as an unwanted consequence of open field production of biologics in food crops (Wolt *et al.* 2004).

Plant pharmaceutical candidates vary from ingestible vaccines to injectible therapeutic antibodies and, therefore, represent equally widely varied understanding regarding the risk associated with a given level of confinement. Therefore, for considerations of open field production, product-specific data on hazard and expression are necessary in addition to estimates of confinement integrity in order that risk be quantitatively described. Here, we consider as a specific instance the case of *Escherichia coli* heat labile enterotoxin subunit B (LT-B) expressed in maize. Maize-expressed LT-B has been investigated in several confined field studies, and both LT-B and maize as a production platform have been widely considered in the literature. Therefore, the exploration and development of this product concept usefully highlights many of the challenges in balancing opportunity and risk within a field of emerging science with significant uncertainties.

Regulatory Background

The regulatory process concerning the safety of plant-manufactured pharmaceuticals (PMPs) involves multiple parties. Under current regulatory statutes in the United States, field experiments with PMPs are assessed by the Department of Agriculture (USDA) through a permitting process that is focused primarily on confinement to limit exposure potential. In the eventuality of commercial production, the Food and Drug Administration (USFDA) subjects the product to assessment under its permitting authority relative to biologics manufacturing. USFDA and USDA have together considered the proposed regulatory needs for production using plant biotechnology systems, including those intended to produce protein antigens (USFDA 2003). Activities are ongoing to refine the regulatory process to better address novel innovations in biotechnology, such as PMPs. Existing guidance provides detailed requirements for assuring safety for the intended uses of plant-produced biologics. In this regard, risks associated with the worker and the intended end-user are (or will be) considered

using well-established frameworks for testing and assessment (IHC 1997). Although much of the requisite data described for manufacturing applications are relevant for the eventual assessment for unintended occurrence in food, there is no explicit guidance for such evaluation. Therefore, addressing risks associated with inadvertent occurrence of open-field produced antigens in food is presently focused on the ability to confine PMPs in experimental trials and eventual manufacturing sites. For the specific case of maize-expressed LT-B, environmental assessments that address confinement have been a condition for permitting of field trials (*e.g.*, see http://www.aphis.usda.gov/brs/aphisdocs/05_06901r_ndd.pdf).

State-of-the-Science for Antigen Expression in Plants

There are substantial advantages for the production of a variety of biologics in plants including low cost of production, rapid scalability, absence of human and animal pathogens, and accurate protein assembly and folding (Ma *et al.* 2003). Technology developers currently are optimizing plant systems for antigen production. For instance, high efficiencies of post-translational glycosylation are sought in plant systems, because this improves pharmacological activity of the expressed protein. High expression levels that are localized to specific plant organs are also sought, so as to achieve needed production efficiencies. And stability to degradation is desired, so that high activity of the antigen can be maintained in storage and processing. For orally administered vaccines, digestive stability is an additional design attribute for the plant-expressed protein, so that high levels of the functional protein will be delivered to sites of action.

In the case of LT-B, which has potential for use as an oral immunogen or adjuvant, either general expression (Streatfield *et al.* 2002) or tissue-specific expression strategies (Chikwamba *et al.* 2002) have been used. Localization of LT-B within seed matrix, especially in the starch endosperm (Chikwamba *et al.* 2003), has imparted considerable process stability because the functional activity of "heat-labile" LT-B expressed in maize kernels is enhanced over that of the native protein (Streatfield *et al.* 2002; Chikwamba *et al.* 2003). In fact, within the maize kernel matrix, LT-B is sufficiently heat-resistant to withstand heat extrusion (temperatures up to 170°C) (Streatfield *et al.* 2002). The apparent starch encapsulation that occurs with kernel-specific expression of LT-B in maize also imparts digestive stability, a favorable attribute for a product such as LT-B that is intended for oral administration. LT-B present in maize meal was shown to be stable when incubated at 37°C in simulated gastric fluid (SGF), whereas soluble LT-B was rapidly digested (Chikwamba *et al.* 2003).

Food crops offer significant advantages for the production of plant-derived antigens. Means are readily available for the processing of food crops. And the processed products—because they have a history of safe use as foods—pose few concerns relative to inadvertent contaminants that could occur in the purified vaccine. Crops such as maize allow for targeted production of antigens in kernels, providing the appropriate biochemical environment for protein accumulation and storage. As dry storage systems at ambient temperatures, kernels allow for production and accumulation of product over long periods without significant loss of activity. Maize is particularly attractive because of high yield, ease of scale-up, amenability for *in vitro*

tissue culture and genetic transformation, and the fact that the kernel matrix carrying the active protein is an incipient for the formulated drug (Ma *et al.* 2003; Peterson and Arntzen 2004). Although currently intended for purification and delivery as formulated compounds, early hope for edible vaccines centered on the possibility for their direct administration in food (Wang *et al.* 2004).

Food Safety Assessment for Transgenic Proteins

Current knowledge regarding transgenic proteins expressed in plants intended for food use helps to frame food safety issues that may pertain to food exposures from antigens expressed in food crops when there is no intention for food use. The requisite information for food safety determinations for transgenic proteins occurring in food crops is relatively well established (ESFA 2004). For instance, insecticidally active Cry proteins expressed in plants through genetic transformation gained their first regulatory approvals for commercial production in the United States in 1996 and therefore have been subject to extensive food safety assessment (USEPA 2001a, b). Common elements in food safety considerations for these transgenic food crops have included allergenic potential, levels and sites of transgenic protein expression, history of protein use, post-translational glycosylation, process and digestive stability of the transgenic protein, protein mammalian functionality and toxicity, and compositional similarity of the transformed plant with comparable foods. Product attributes for PMPs, such as antigens, are not necessarily consistent with expectations for safety of food proteins (Table 1); therefore, risk assessments for inadvertent occurrence of plant-derived protein antigens in food must recognize the potential for exposure as a mitigating factor.

The Need for a Risk Assessment Paradigm for Inadvertent Occurrence of Plant-Based Antigens in Food

Within the United States inadvertent exposure from transgenic protein in food involves product recall. This process has been invoked in the cases where transgenic proteins have inadvertently entered the food supply and where, because no tolerance or exemption from tolerance in food was established, there was no recourse but product recall (Bucchini and Goldman 2002; Macilwain 2005). Retrospective food safety assessments conducted following evidence for the inadvertent occurrence of Cry9C in foodstuffs, proved inadequate to address the issue of food adulteration with a protein for which no tolerance existed (Petersen *et al.* 2001; Bucchini and Goldman 2002). Unlike the cases of food adulteration involving the unapproved events StarLink and Bt10, production of plant biogenic agents—such as a plant-based antigen—entails strict controls over production, channeling, and disposition of the product as well as a very limited extent of production. There is no process for tolerance establishment or exemption for these products. Nonetheless, there are instances of potential introduction of PMPs from confined field trials into the food supply (USDA 2002). Understanding the food safety ramifications of such inadvertent instances of PMP introduction to the food supply help to clarify the degree of risks such instances pose to consumers.

Table 1. Comparison of common food safety attributes of a plant transformed to express an insect resistance protein as compared to product concepts for a food crop expressing a pharmaceutically active protein.

Product attribute	Food Crop Food safety expectation for a plant-made insecticide	Pharma Crop Current product concepts for a plant-made antigen
Allergenic potential	No homology to known allergens	Allergenic compounds managed to control exposure
Expression	Low to negligible in processed food fraction	Maximize in an readily processed food fraction
Familiarity	History of human dietary exposure to the expressed product	varies
Food tolerance	Exempt from tolerance	No tolerance
Functionality	Loss of activity in processing	Stable to processing
Glycosylation	Absence of post-translational glycosylation	Utilize the plant system to maximize potential for post-translational glycosylation
Heat stability	Loss of activity at moderate temperatures over short periods	Stable to heat extrusion temperatures
Mammalian activity	Non-active	Highly active
Pepsin digestion	Must be rapidly degraded in simulated digestion fluid	Digestive stability is preferred (in the case of an oral antigen)
Substantial equivalence	An existing organism used as food with a history of safe use, can serve as a comparator when assessing the safety of the genetically modified food	An existing organism used as food with a history of safe use, can serve as a comparator when assessing the safety of the pharmaceutical purified from the food crop

INADVERTENT DIETARY EXPOSURE ASSESSMENT FOR LT-B

Addressing food safety concerns for inadvertent exposure to a plant-produced antigen not intended for occurrence in food entails a consideration of the inherent hazard of the biogenic agent and, secondly, an understanding of the levels of exposure that may occur in food. Together, hazard and exposure will inform the characterization of risk posed by the biogenic agent via dietary routes. Fortunately, much of the requisite data for the risk characterization and food safety determination are developed to meet existing criteria addressing intended uses of plant-derived pharmaceuticals (USFDA 2003). We evaluate here various food safety decision criteria and describe their application to maize-expressed LT-B.

Functionality and Toxicity Profile

The data developed for a plant-based antigen will provide a profile of protein functionality and toxicity that will serve as a good starting point for a food safety evaluation. The data developed from current guidance (USFDA 2003) should allow the risk assessor to address: What is the nature and site of action? What are the common immunizing doses and dosing regimes? What adverse affects have been reported and under what conditions and doses? However, because short-term intake via oral ingestion represents the salient route of exposure when considering indirect, inadvertent occurrence of a plant-based antigen in the diet, appropriate data relating to this food safety consideration may be lacking.

In the case of LT-B the established understanding is that, as a non-pathogenic subunit of the multiunit heat-labile enterotoxin (LT), it affects the binding of the toxin to the host cell receptor. LT-B itself is strongly immunogenic (Dickinson and Clements 1996) and may additionally have an adjuvant effect in stimulating immune responses to co-administered antigens (Millar *et al.* 2001). Because LT-B is a potent antigen with significant adjuvant activity, it may be possible to prime the body at levels of exposure well below the dose conferring immunogenicity (J. Cunnick, personal communication).

There is no evidence of LT-B toxicity under projected conditions of use from either *in vivo* or *in vitro* studies. The patent-mouse assay has been used to measure *in vivo* effects of LT-B (Guidry *et al.* 1997). This assay measures water leakage from tissues and accumulation of fluid within the lumen of the intestine following acute exposure. Intragastric administration of immunogenically significant amounts of bacterial LT-B (25 μg and 125 μg) in buffer showed no difference in effect as compared to buffer-only controls (Guidry *et al.* 1997). This *in vivo* result is consistent with *in vitro* tests that show no effect as compared to appropriate controls for measurements of Y-1 adrenal cell activity (Guidry *et al.* 1997; Cheng *et al.* 2000) as well as in a Chinese hamster ovary cell assay, rabbit ileal loop test, and vascular permeability test (Takeda *et al.* 1981).

The nature of LT-B interaction with the immune system, however, is not presently understood and ongoing research seeks to determine whether indirect toxicity through an adjuvant effect on a toxic protein is possible (Williams 2000). Until the nature of LT-B effects is better understood, caution is warranted relative to the unintended occurrence of LT-B in food.

Acute toxicity testing using a dosing stratagem consistent with dietary exposure is not the norm for a vaccine. In toxicity testing of a biopharmaceutical, "The route and frequency of administration should be as close as possible to that proposed for clinical use" (page 4, IHC 1997). Therefore, antigen testing for adverse effects is apt to occur within the range of the immunogenic dose and the pattern of clinical use will not necessarily establish the toxicological profile nor reflect patterns of indirect exposure through adulterated food consumption.

In the case of LT-B, toxicity tests provide data that can conservatively bridge to food safety considerations. Adult female mice (representative body weight of 30 g) showed a no observed adverse effect level (NOAEL) when administered 125 μg of LT-B (Guidry *et al.* 1997). Thus, the acute oral toxicity endpoint for LT-B is > 0.042 mg kg^{-1} when including 2 10-fold uncertainty factors to account for interspecies and intraspecies population extrapolation (Kodell and Gaylor 1999). This endpoint appears reasonable on the basis of a lack of adverse effects in clinical trials with humans who ingested up to 1.1 mg LT-B from transgenic potato (approximately 0.016 mg kg^{-1} body weight for a mean adult body weight of 70 kg; Tacket *et al.* 1998).

Allergenicity

Allergenicity is a special food safety concern when novel proteins are introduced into the diet. In assessing the risk, relevant questions are, therefore: Is the protein known to cause allergenic reactions? Does it share homology with known allergens? Is the source of the transgenically expressed protein known to be allergenic? In the case of food proteins, homology with known allergens (an 8-monomer match along any contiguous portion of the protein; ESFA 2004) heightens concerns sufficiently to preclude regulatory acceptance. (This is because robust animal models to test for allergens are not presently available.) A search of the maize-expressed LT-B amino acid sequence against a database of known protein allergens following recognized sequence evaluation schemes (Gendel 1998; FAO 2001) showed no matches for 35% or greater homology over an 80 amino acid window and no match of any 8 contiguous amino acids (P. Song, personal communication). Although LT-B does not share sequence homology with known allergens, there exist the aforementioned uncertainties regarding unintended adjuvant effects to heighten response to allergens in the diet.

Protein Stability

Loss of protein function, especially loss due to degradation of the protein, is an effective means to mitigate risks due to inadvertent dietary exposure. Additionally, stability is a characteristic differentiating allergenic protein from proteins in general (Bannon *et al.* 2003). Important food safety questions are, therefore: Is the protein heat labile? Will the protein degrade in processing or in the digestive tract? Do the relevant studies describe only loss of functionality or do they show physical degradation of the protein?

For maize-expressed transgenic Cry proteins registered to date, digestion studies using pepsin to simulate gastric fluid have shown loss of 90% of detectible protein (DT_{90}) in less than 7 minutes (USEPA 1998; Lamphear *et al.* 2002). As described earlier, LT-B contrasts with these Cry proteins in that the plant-derived LT-B,

especially in the matrix of maize seed, is stable to the high temperatures experienced in protein extrusion typical of food production processes such as cereal manufacture and is apparently stable under other conditions of processing as well (Streatfield *et al.* 2002). In addition, the plant-produced protein is not readily degraded in SGF (protein remains detectable after 15 minutes); whereas, soluble bacterial LT-B degrades in SGF in less than 5 minutes (Chikwamba *et al.* 2003). The distinction in digestibility between the Cry proteins and LT-B is not clear-cut, however, because stability and degradation studies for Cry proteins typically are conducted with the microbially expressed counterpart to the plant-expressed protein. Consequently, the effect of plant matrix, which has been shown in the case of LT-B (Streatfield *et al.* 2002; Chikwamba *et al.* 2003), has not been commonly addressed for the Cry proteins. Regardless, the stability of LT-B is such that processing, preparation, and digestion would not mitigate exposure to plant-derived LT-B inadvertently present in food.

Expression Level

Current food safety evaluations for transgenic proteins seek to understand levels of expression and protein distribution within various parts of the plant. This information is needed to determine dietary exposure as part of the food tolerance assessment. The food safety determination, thus, considers the maximum anticipated level of expression in consumable fractions of the crop and how expression varies due to plant genetic and environmental factors. For maize-expressed Cry proteins, expression in kernels is in the <1 to 10 $\mu g\ g^{-1}$ range (USEPA 2001a, b). For maize-derived LT-B, kernel expression levels of 9.2% of total soluble protein (TSP; Streatfield *et al.* 2002), and 3.7% of TSP have been achieved using strong or seed-specific promoters (Chikwamba *et al.* 2002, 2003). As much as 350 $\mu g\ g^{-1}$ LT-B could be obtained in kernels with a seed-specific promoter (Chikwamba *et al.* 2002).

Equivalence

Equivalence of both plant hosts and plant-expressed transgenic proteins to their common counterparts found in commerce is an important aspect of safety evaluations for both foods and vaccines. Equivalence considerations for a vaccine are focused on the administration of the agent; whereas equivalence from a food safety perspective is focused on food consumption. Recognition of the somewhat differing needs for these considerations can lead to improved strategies within existing guidance (USFDA 2003) to address both aspects of equivalence. In terms of food safety considerations, key questions are: Has the equivalence of the plant-expressed agent to the currently commercial forms (or forms used in toxicity testing) been established? Are these data based on bioactivity measures and/or physical measurements? And, is the host plant composition substantially the same as the currently used products?

For maize-expressed LT-B, data establish equivalence to microbial forms in terms of activity and physical characteristics (Chikwamba *et al.* 2002; Lamphear *et al.* 2002) and USFDA guidance dictates establishing compositional equivalence of the transformed host as part of the manufacturing application (USFDA 2003).

Glycosylation

Post-translational glycosylation is a concern for transgenic proteins in food because this may confer potential immunogenicity or allergenicity (Samyn-Petit *et al.* 2001). Even though plant glycoproteins are ubiquitous in the human diet, the glycosylation of a plant transgenic protein to produce a novel glycoprotein needs to be considered. With regard to Cry proteins in plants, glycosylation is considered from the standpoint of altered activity that may be of consequence to specificity and functionality of the transgenic protein. For plant-produced biologics the consideration of glycosylation needs to be broadened to address the nature of the glycosylation that may occur because it may confer benefits to the activity of the agent without negative ramifications relative to adverse immunogenic or allergenic effects. Post-translational glycosylation of plant-made LT-B has not been reported.

Dietary Exposure

Quantitative dietary exposure assessment is a means to evaluate the relevance of the forgoing food safety considerations with respect to unintended presence of plant-based vaccines in food. Again, existing approaches demonstrate how quantitative exposure assessment may be usefully applied to PMPs, including plant-made antigens. Using a conservative estimate of Cry protein in maize (10 μg mg^{-1}; USEPA 2001b), a high-end consuming subpopulation (95th percentile consumers, Table 2) could be potentially exposed to 700 mg Cry protein per day, if Cry protein were present in 80% of consumed maize food forms. (In 2005, 35% of maize planted in the United States was from varieties expressing Cry proteins for insect resistance, but local instances approaching 80% do occur; NASS 2005.) This estimate does not account for the fact the Cry proteins will be unstable due to processing, preparation, and digestion; thus, actual dietary exposure from the Cry proteins in food may be negligible.

Table 2. Consumption of maize food products (gram per capita per day) by the U.S. population and selected subpopulations.[a]

	50th percentile	95th percentile
U.S. population	5.1	62.4
All infants	2.3	11.6
Children 1–6 years	6.0	41.2
Children 7–12 years	9.5	61.9
Females 13–19 years[b]	4.1	55.2
Males 13–19 years	7.2	85.9
Females 20+ years	3.2	44.0
Males 20+ years	6.7	83.8

[a]Reflecting total daily consumption of maize food and food forms (flour, meal, bran, and starch) determined from the Continuing Survey of Food Intake by Individuals (HNRC 1998; USEPA 2003).
[b]Not pregnant or lactating.

In comparison, Wang *et al.* (2004) estimate that with current levels of endosperm-specific LT-B expression in maize (350 μg g^{-1}), as little as 3 g maize meal will constitute a functional dose of LT-B sufficient to elicit a protective immune response in humans (1.1 mg). Because we lack a complete toxicology profile for LT-B, hazard is not established; therefore, we use the immunizing dose as a conservative measurement endpoint. Because the transgenic protein expression level is likely to increase due to further technology improvement, we reasonably project that line improvement will result in commercial production of LT-B maize where as little as 0.2 g maize endosperm (a 15-fold increase over current expression levels) will constitute a functional dose. Using this estimate of a functional dose as a measurement endpoint, we can address through quantitative exposure assessment: What is the level and significance of LT-B which may inadvertently occur in food?

To do this we advance three scenarios that reflect various possibilities for adulteration of food from plant-expressed LT-B that may occur through various degrees of handling error (Figure 1). In each case, we are concerned with the high-end (95th percentile) daily consumption (86 g maize) for the highest consuming subpopulation (males 13 to 19 years old; Table 2). For this most exposed population, foods containing maize would need to be 0.25% adulterated with maize-derived LT-B to result in a functional dose (see later example). Because the commercial production of LT-B maize will involve a few locations of limited extent, adulteration of foods at these levels would be a very unlikely occurrence. However, we consider here scenarios involving a one time event where LT-B maize is inadvertently processed through a dry mill into tortilla chips. These scenarios assume that the functionality of LT-B is not lost in the alkaline cooking process. We further assume that tortilla chips represent the sole source of daily maize consumption by high-end consumers (about 3 servings of tortilla chips, a reasonable assumption for 13- to 19-year-old males).

Our first scenario considers an extreme worst case that provides an upper bound of concern for the inadvertent introduction of the plant-based antigen into the food supply. The extreme worst case involves an event where one hectare of LT-B expressing maize (this represents about 3/4 of the hopper capacity of a production combine) is harvested and inadvertently directed and processed into tortilla chips. About 2% of typical U.S. maize production is processed into cereal products (Ag MRC 2003); therefore, this exposure scenario for regulated biogenic maize is rather unlikely. Nevertheless, the case is analogous to what has occurred with introduction of StarLink and Bt10 maize into the food supply; albeit at a much more restricted scale that reflects the size of open-field plant-based antigen production.

We assume that the LT-B expression level in maize kernel is 5 mg per gram of dry grain. The scenario considers that one hectare of LT-B maize will yield 5 Mg (a reasonable estimate based on yields for unimproved varieties of maize; Wang *et al.*, unpublished). We further assume that all 5 Mg of LT-B grain are mistakenly directed to a dry mill with a daily milling capacity of 2000 Mg (AgMRC 2003) where the grain is processed into tortilla chips. This has at worst a 1 in 50 chance of occurring because less than 2% of maize will be processed for this use. In this case, the concentration of LT-B maize adulterating the maize processed into tortilla chips is 0.25% $[= 100 \times 5/(2000 - 5)]$. Assuming that a high-end consumer in the most exposed group consumed 86 g maize as tortilla chips per day (Table 2), the result is ingestion of 0.215 g of LT-B maize ($= 86 \times 0.25\%$). Because we projected that the LT-B

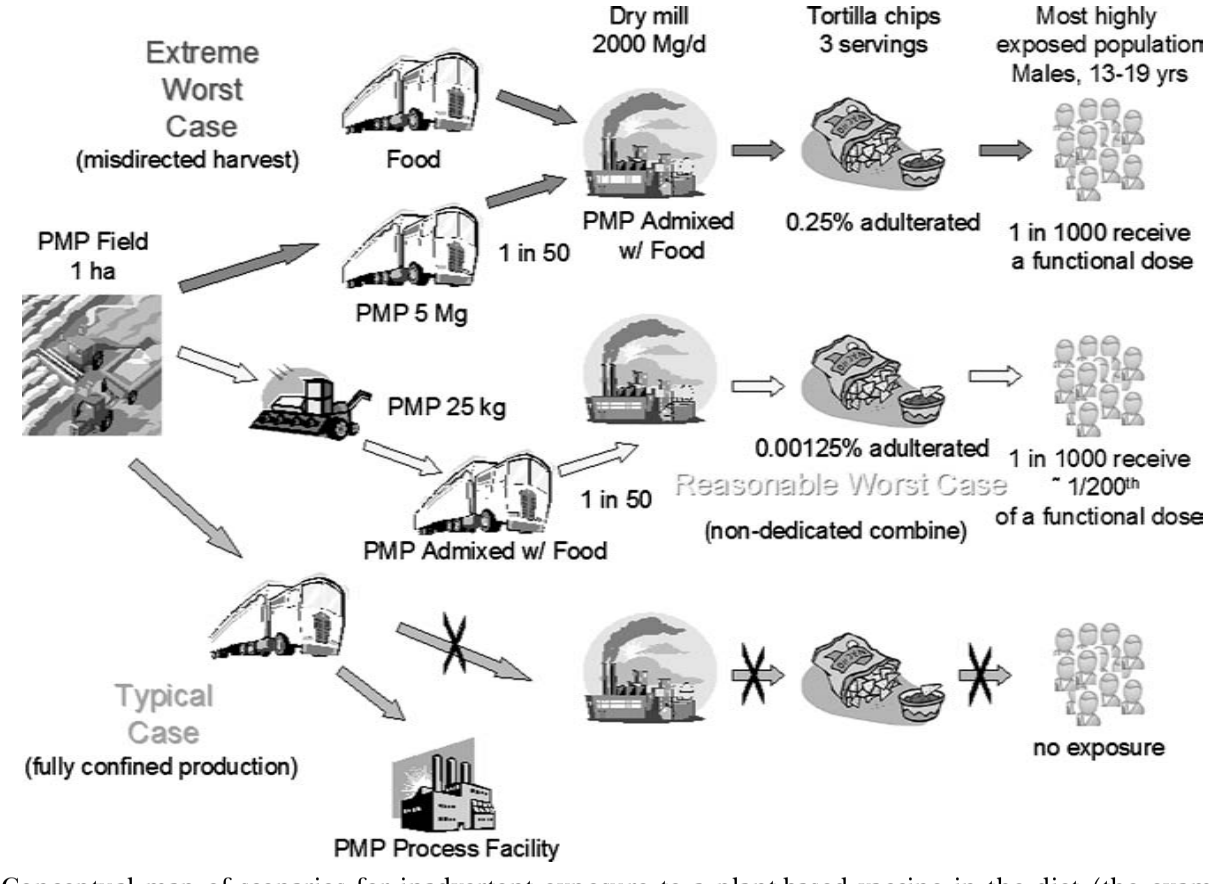

Figure 1. Conceptual map of scenarios for inadvertent exposure to a plant-based vaccine in the diet (the example assumes LT-B maize expressing 0.5% in seed endosperm). The extreme worst case represents a screening scenario that sets an upper limit for potential risk based on a catastrophic scenario; whereas, the reasonable worst case represents high-end exposures due to inappropriate harvester clean-out. The typical case represents a properly managed production system.

expression level was 5 mg g^{-1} seed, the level of LT-B ingestion is 1.1 mg (= 0.215 g × 5 mg g^{-1}), equal to the functional dose of LT-B (Tacket *et al.* 1998). Moreover, the high end consumers represent five in 100 consumers (95th percentile) in the most exposed group and the adulterated chips come from a mill that represents 1/50th of total daily maize grind capacity for the United States (AgMRC 2003). Thus, 1 in 1000 consumers (= 5/100 × 1/50) in the most highly exposed population could receive a functional dose of LT-B by consuming 3 or more servings of tortilla chips (a typical serving for tortilla chip consists of 13 chips). This example does not take into account that the maize process fractions can be further refined for oil removal and the resulting defatted germ fraction may be as much as 8-fold concentrated in LT-B because of process stability (Lamphear *et al.* 2002). However, the consumption of such a product in human diets is very limited.

Because confinement and channeling of the plant-produced biogenic agents minimizes the potential for the extreme worst case exposure, a refined consideration using a reasonable worst scenario is helpful for better understanding exposure potential. For the reasonable worst case we posit that an admixture of the biogenic grain to food grain occurs from improperly used and cleaned harvest equipment (a one-time introduction of 25 kg biogenic grain to the food supply; Wolt *et al.* 2004). In this instance, potential exposure to the high-end consumer would be about 200-fold below a functional dose of LT-B (admixture of 25 kg LT-B maize in the 2000 Mg daily processing of a mill and assuming a functional dose constitutes 0.215 g LT-B maize). And, as with the extreme worst case scenario, this level of potential exposure is restricted to a small fraction of high-end consumers. Exposure relative to functional dose under this scenario is effectively zero and should not be construed as adulteration.

Finally, we need to bear in mind that the typical case exposure to the general food supply is negligible when the plant-made antigen is confined and channeled in accordance to experimental permit or manufacturing license.

DISCUSSION

Food safety issues relative to the unintended presence of a plant-made antigen in food can best be approached through the formalized process of risk assessment. Risk assessment involves the scientific evaluation of hazard (the potential of an identified source to cause an adverse effect) in conjunction with realistic estimates of exposure. This process proceeds in sequential steps to "identify characteristics that may cause adverse effects, evaluate their potential consequence, assess the likelihood of occurrence and estimate the risk posed by each identified characteristic" (EFSA 2004). This process is readily applied to food safety considerations for genetically engineered food crops (EFSA 2004; EC 2000), and by extension is the appropriate means whereby the risks posed by production of plant biogenic agents should be evaluated (Peterson and Arntzen 2004). The risk assessment paradigm answers the salient questions within a comprehensive risk analysis process that is science-based (Wolt and Peterson 2000; Howard and Donnelly 2004; Peterson and Arntzen 2004).

We have shown here that a weight-of-evidence approach to assessing food safety for unintended occurrence of a biogenic agent in food can have short-comings if

hazard data gaps exist. However, when there is relevant knowledge of toxicity and protein fate through inadvertent exposure, as is the case for LT-B, it is possible to quantitatively evaluate the food safety implications arising from inadvertent occurrence in food from the risk-based consideration of exposure as balanced against effect. For instance, stability of the LT-B protein does not seem relevant to food safety considerations because the opportunity for—and level of—inadvertent exposure is negligible and other protein attributes that could pose toxicity or allergenicity concerns are absent.

As we have shown, exposures through inadvertent presence can be estimated, but when there is uncertainty with respect to hazard leading to an expectation of zero tolerance, the risk assessment can be inconclusive. For other plant-derived proteins, the ability to bridge from clinical data to estimates of dietary risk may be more difficult than is the case of LT-B because of a lack of relevant toxicological data. The need for vaccine toxicity testing goes beyond considerations of plant biogenic agents. There is an emerging paradigm for vaccine safety assessment that more strongly reflects toxicological considerations (SOT 2002) and this will help to the degree that guidance for test design extends to consideration of dosing regimes consistent with inadvertent occurrence in foods.

We have focused here on extending principles of food safety assessment to a product that is never intended to occur in food. Acute exposure through product mishandling at harvest is the relevant consideration based on potential routes and quantities of exposure due to confinement loss (Wolt *et al.* 2004). Alternative exposure routes such as pollen drift from confined fields necessitate a somewhat differing perspective on the nature of harm that may be manifested; but as with the present analysis, exposure potential limits the opportunity for an adverse health consequence (Howard and Donnelly 2004). Success in continued development and future production of plant-expressed biologics under field confinement will depend on recognition and adoption of risk-based approaches for determination and assessment of inadvertent occurrence in foods.

ACKNOWLEDGEMENTS

We thank P. Song for conducting the allergen homology search for LT-B. This work has been supported through a grant from the Iowa State University Plant Sciences Institute.

REFERENCES

AgMRC (Agricultural Marketing Resource Center). 2003. The U.S. Corn Masa Industry: Structure and Implications for the Great Plains Region. Prepared for AgMARC by Sparks Companies, Inc., Department of Agricultural Economics, Kansas State University, Manhattan, KS, USA

Bannon G, Fu T-J, Kimber I, *et al.* 2003. Protein digestibility and relevance to allergenicity. Environ Health Persp 111:1122–4

Bucchini L and Goldman LR. 2002. Starlink maize: A risk analysis. Environ Health Persp 110:5–13

Cheng E, Cárdenas-Freytag L, and Clements JD. 2000. The role of cAMP in mucosal adjuvanticity of *Escherichia coli* heat-labile enterotoxin (LT). Vaccine 18:38–49

Chikwamba RK, McMurray J, Shou H, *et al.* 2002. Expression of a synthetic E. coli heat-labile enterotoxin B sub-unit (LT-B) in maize. Molec Breed 10:253–65

Chikwamba RK, Scott MP, Meja LB, *et al.* 2003. Localization of a bacterial protein in starch granules of transgenic maize kernels. Proc Nat Acad Sci USA 100:11127–32

Christensen P, Misra MK, Rai S, *et al.* 2005. Confined Production Processes for Non-Food Corn. Biosafety Institute for Genetically Modified Agricultural Products, Iowa State University, Ames, IA, USA

Dickinson BL and Clements JD. 1996. Use of *Escherichia coli* heat-labile enterotoxin as an oral adjuvant. In: Kiyono H, Ogra PL, and McGhee JR (eds), *Mucosal Vaccines.* pp 73–87. Academic Press, Inc., San Diego, CA, USA

EC (European Commission). 2000. Report of the Scientific Steering Committee's Working Group on Harmonisation of Risk Assessment Procedures in the Scientific Committees advising the European Commission in the area of human and environmental health—26–27 October 2000. European Commission Directorate-General Health & Consumer Protection, Brussels, Belgium

EFSA (European Food Safety Authority). 2004. Draft Guidance Document for the Risk Assessment of Genetically Modified Plants and Derived Food and Feed, April 2004. European Food Safety Authority, Brussels, Belgium

FAO (Food and Agriculture Organization). 2001. Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology, 2001. Evaluation of allergenicity of genetically modified foods. 22–25 Jan 2001. FAO/WHO, Rome, Italy

Gendel S. 1998. The use of amino acid sequence alignments to assess potential allergenicity of proteins used in genetically modified foods. Adv Food Nutr Res 42:45–62

Guidry JJ, Cardenas L, Cheng E, *et al.* 1997. Role of receptor binding in toxicity, immunogenicity, and adjuvanticity of *E. coli* heat labile enterotoxin. Infect Immun 65:4943–50

HNRC (Human Nutrition Research Center). 1998. Continuing Survey of Food Intakes by Individuals (CSFII) 1994–96, 1998 Data File. NTIS PB2000-500027. USDA Agricultural Research Service, Beltsville, Maryland, USA

Howard JA and Donnelly KC. 2004. A quantitative safety assessment model for transgenic protein products produced in agricultural crops. J Agric Environ Ethics 17:545–58

IHC (International Conference on Harmonisation). 1997. Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals, ICH Harmonised Tripartite Guideline S6, 16 July 1997. ICH Secretariat, Geneva, Switzerland

Kodell RL and Gaylor DW. 1999. Combining uncertainty factors in deriving human exposure levels of noncarcinogenic toxicants. Annals NY Acad Sci 895:188–95

Lamphear BJ, Streatfield SJ, Jilka JM, *et al.* 2002. Delivery of subunit vaccines in maize seed. J Controlled Release 85:169–80

Ma JK-C, Drake PMW, and Christou P. 2003. The production of recombinant pharmaceutical protein in plants. Nature Reviews Genetics 4:794–805

Macilwain C. 2005. US launches probe into sales of unapproved transgenic corn. Nature 434:423

Millar GG, Hirst TR, and Snider DP. 2001. *Escherichia coli* heat-labile enterotoxin B subunit is a more potent mucosal adjuvant than its closely related homologue, the B subunit of cholera toxin. Infection Immun 69:3476–82

NASS (National Agricultural Statistics Service). 2005. Crop Production Acreage Supplement. 30 June 2005. Available at http://usda.mannlib.cornell.edu/reports/nassr/field/pcp-bba/acrg0605.txt

Petersen BJ, Rachman, NJ, and Watters JL. 2001. Estimated Potential Dietary Intake of Cry9c Protein Based on Measurements of Cry9c In Processed Foods Made from 100% Starlink™

Corn. Available at http://www.epa.gov/pesticides/biopesticides/pips/old/stlink/starlink_01-01_86-5_report_final.pdf

Peterson RKD and Arntzen CJ. 2004. On risk and plant-based biopharmaceuticals. Trends Biotechnol 22:64–6

Samyn-Petit B, Gruber V, Flahaut C, *et al.* 2001. N-glycosylation potential of maize: The human lactoferrin used as a model. Glycoconjugate J 18:519–27

SOT (Society of Toxicology). 2002. Proceedings of the Workshop on Non-clinical Safety Evaluation of Preventive Vaccines: Recent Advances and Regulatory Considerations. Available at http://www.toxicology.org/ai/meet/cct-vaccines.asp

Streatfield SJ, Mayor JM, Barker DK, *et al.* 2002. Development of an edible subunit vaccine in corn against enterogenic strains of *Eschericia coli*. In Vitro Cell Dev—Plant 38:11–17

Tacket CO, Mason HS, Losonsky G, *et al.* 1998. Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. Nature Medicine 4:607–9

Takeda Y, Honda T, Taga S, *et al.* 1981. In vitro formation of hybrid toxins between subunits of *Escherichia coli* heat-labile enterotoxin and those of cholera enterotoxin. Infection Immun 34:341–6

USDA (United States Department of Agriculture). 2002. USDA investigates biotech company for possible permit violations. Available at http://www.aphis.usda.gov/lpa/news/2002/11/prodigene.html

USEPA (United States Environmental Protection Agency). 1998. *Bacillus thuringiensis* Subspecies *tolworthi* Cry9C Protein and the Genetic Material Necessary for its Production in Corn; Exemption from the Requirement of a Tolerance Related Material. Federal Register 63:28258–61

USEPA (US Environmental Protection Agency). 2001a. Pesticide Fact Sheet: *Bacillus thuringiensis* subspecies Cry1F Protein and the Genetic Material Necessary for Its Production (Plasmid Insert PHI 8999) in Corn. EPA Office of Prevention, Pesticides, and Toxic Substances, Washington, DC, USA

USEPA (US Environmental Protection Agency). 2001b. Biopesticides Registration Action Document—*Bacillus thuringiensis* Plant-Incorporated Protectants. EPA Office of Prevention, Pesticides, and Toxic Substances, Washington, DC, USA

USEPA (US Environmental Protection Agency). 2003. CSFII Analysis of Food Intake Distributions, EPA/600/R-03/029. EPA Risk Assessment Forum, Washington, DC, USA

USFDA (US Food and Drug Administration). 2003. Guidance for Industry: Drugs, Biologics, and Medical Devices Derived from Bioengineered Plants for Use in Humans and Animals, Draft Guidance. FDA Center for Biologics Evaluation and Research, Washington, DC, USA

Wang K, Chikwamba R, and Cunnick J. 2004. Plant-based oral vaccines. Ch. 18 In: Kaufmann SHE (ed.), *Novel Vaccination Strategies*. Wiley-VCH Verlag, Weinheim, Germany

Williams NA. 2000. Immune modulation by the cholera-like enterotoxin B-subunits: From adjuvant to immunotherapeutic. Intl J Med Microbiol 290:447–53

Wolt JD and Peterson RKD. 2000. Agricultural biotechnology and societal decision-making: The role of risk analysis. AgBioForum 3:291–8

Wolt JD, Shyy Y-Y, Christensen P, *et al.* 2004. Quantitative exposure assessment for confinement of maize biogenic systems. Environ Biosafety Res 3:183–96