RISK ASSESSMENT OF PLANT-BASED

PHARMACEUTICALS AND BIOLOGICS

by

Leslie Marie Shama

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APPROVAL

of a thesis submitted by

Leslie Marie Shama

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the Division of Graduate Education.

Dr. Robert K.D. Peterson

Dr. Andreas Fischer

Approved for the Department of Plant Sciences and Plant Pathology

Dr. John Sherwood

Approved for the Division of Graduate Education

Dr. Carl Fox

STATEMENT OF PERMISSION TO USE

Leslie Marie Shama

November 2006

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ABSTRACT

Biotechnology is evolving to produce pharmaceutical proteins in plants. Plant-based pharmaceutical production creates concerns of exposure in an open environment and contamination of the food supply. Consequently, quantitative human health and ecological risk assessments were conducted for aprotinin, gastric lipase, and LT-B expressed in maize. A comparative, qualitative risk assessment was conducted for conventionally derived and plant-cell derived Newcastle disease virus vaccine. Effect and exposure scenarios were modeled for each quantitative risk assessment and each scenario was based on a tiered approach in which inadvertent exposure through ingestion were examined to determine a risk characterization of the plant-based pharmaceuticals. The qualitative Newcastle disease virus vaccine risk assessment characterized risks based on the potential exposures to the poultry being vaccinated, to humans administering the vaccine, and to non-target birds. For the human-health risk assessment, the dietary exposure evaluation model (DEEM) was used to estimate the inadvertent dietary intake of the pharmaceutical proteins in food. The ecological risk assessment used Monte Carlo simulations to evaluate the exposure of each protein in maize for four receptor species. The human-health risk assessments revealed that the most conservative scenario produced higher risk quotients (RQ's) than the other two scenarios. The difference in risks was attributable to the differences in toxic endpoints of the proteins. Although the protein expressions and dietary consumption were assumed to be the same, dietary risks between the proteins varied by more than 56,000 times. The human-health risk assessment revealed that risks will vary dramatically and depend on factors such as the specific pharmaceutical protein, protein expression, and exposure scenarios. The assessments reinforced the need for case-by-case assessments. The ecological risk assessment demonstrated that risks will vary between species and between proteins, based primarily on differences in toxic endpoint and consumption rates. It shows the utility of probabilistic, quantitative risk assessment methodologies and supports the human dietary risk assessment by demonstrating the importance of assessing risks from plant-based pharmaceuticals on a case-by-case basis. The qualitative assessment illustrated that fewer effects and contamination issues were associated with the use of the plant-cell derived Newcastle disease virus vaccine compared to conventionally derived vaccines.

CHAPTER 1

INTRODUCTION

Pharmaceuticals using genetically engineered proteins have been made through protein expression in bacterial, fungal, and mammalian cell cultures. Biotechnology is evolving to produce more complex and diverse pharmaceutical proteins in plants. The pharmaceuticals are known as plant-made or plant-based pharmaceuticals.

Genetic engineering has made it possible to use plants as factories for pharmaceutical protein production. Plant-based pharmaceuticals are made by inserting a segment of DNA that encodes the protein of choice into the plant cells. The plants or the plant cells are essentially factories used to produce the desired proteins and are only grown for the purpose of pharmaceutical applications (Shama and Peterson 2004).

Many people suffer from infectious, inflammatory, and cardiovascular diseases—and these numbers are growing. Protein-based drugs are the fastest growing class of drugs for the treatment of these diseases in humans and other animals. The current method of producing proteins for pharmaceutical application is predicted to fall short in the years to come because of population growth and demographic trends (Rogers 2003).

Plant-based pharmaceutical production creates concerns of exposure in an open environment and contamination of the food supply. Revisions of regulatory guidelines are currently being made to address the differences in protein production for pharmaceutical use and the concerns of contamination both in the environment and in our food.

For each protein that is inserted through biotechnological methods into a plant there will be different results. Proteins all have different structures, stabilities, and over-all characteristics and because of this, regulatory guidelines should address each plant/protein combination differently. By using a risk based approach to address these concerns, and others many of the implications can be resolved or managed by knowing where the risk occurs in the system.

The traditional method of vaccine production was contained in facilities where the cell cultures were grown and not exposed to the open environment. With this new method of "farming" for proteins, they will be exposed to the environment. However, in a field there are special containment practices that can be employed to reduce the risk of contamination by the transgenic plants to non-transgenic plants. Risks pertaining to this method of producing pharmaceutical proteins will need to be evaluated on a case-by-case basis to ensure proper precautions are made prior to releasing these plants into the environment.

Plant Options

Plant-made pharmaceutical production has the potential to provide large amounts of protein for the pharmaceutical industry. The types of plants used for pharmaceutical protein production depend largely on their final application. To date, the plants that have successfully been transformed include tobacco, potato, tomato, corn, soybean, alfalfa, rice, and wheat. However, it is important to emphasize that no commercially available pharmaceutical products are currently produced in plants (Ma et al. 2003).

Tobacco was the first plant to be genetically engineered and has the advantage of being used as a plant biopharmaceutical for exactly that reason; the methods for gene transfer and expression are well established for this plant. Tobacco can also be cropped multiple times per year, giving it an edge over other plants in producing a large biomass (Fischer et al. 2003). The protein of interest can also be targeted to the seed. In tobacco, up to one million seeds can be made on a single plant (Daniell et al. 2001). One concern associated with using tobacco as a protein factory is that it has toxic alkaloids associated with it, which could be present in the final drug that is administered to the patient. Another concern is that if the protein is targeted for the leaf tissue, it must be frozen or dried and then processed immediately, making it difficult to harvest and store for preservation of the protein (Ma et al. 2003).

Legumes, such as alfalfa and soybean, and cereal crops, such as corn and rice, have been considered as ideal candidates for plant biopharmaceuticals because the protein can be targeted to accumulate in the seed and the seed can be harvested and stored for an extended amount of time. Legumes are good candidates because they naturally produce large amounts of protein in their seeds, making the final protein recovery much larger. When considering a cereal crop as a protein factory, yield is the main quality of importance, as well as ease of transformation and speed of production scale-up (Ma et al. 2003).

<div align="center">Protein Application</div>

The application of the proteins made in plants includes antibodies, vaccines, hormones, enzymes, interleukins, interferons, and human serum albumins. Monoclonal

antibodies have the potential to be among the first protein pharmaceuticals commercially produced in plants (Rogers 2003). Antibodies are produced in vertebrate immune systems to recognize and bind to antigens with amazing specificity. Because antibodies possess this specificity, they can be used as diagnostic tools as well as for prevention and treatment of diseases (Fischer et al. 2003). There are more than 10 plant-derived recombinant antibodies that have been clinically validated and almost 1000 therapeutic antibodies being tested for various disorders and diseases (Gomord et al. 2004). A possible future use for monoclonal antibodies is to attach a chemotherapeutic agent onto the antibody and allow it to specifically bind to a tumor cell and eventually the chemotherapeutic agent will destroy the tumor cell (Rogers 2003).

Another application for plant-made pharmaceuticals is the production of vaccines. Subunit vaccines are made up of specific macromolecules that induce a protective immune response against a pathogen. Subunit vaccine technology has increased the safety of administering vaccines because it does not involve the use of live or weakened viruses. Currently, it is very expensive to produce subunit vaccines as well as to store them because they are not heat-stable. Because the vaccines are not heat-stable, this limits where they can be sent for use and unfortunately makes them unavailable in the developing countries where vaccines are needed the most. Producing the vaccines in plants eliminates the heat-stability issue because the vaccinogenic plant tissue can be administered raw, dried, or in an encapsulated form; all of these forms can be stored and shipped at room temperature (Sala et al. 2003). The risk of contamination with animal pathogens during production is also eliminated (Walmsley et al. 2000). Oral

delivery of the vaccine is another reason that vaccines produced in plants are an attractive possibility because they can eliminate injection-related risks. Overall, the fact that the vaccines can be stored as seeds are advantageous because large amounts of vaccines can be produced in limited time and storage is less of an issue because the seed is a stable form that will not degrade the protein over time.

The choice of the plant species will determine the way the vaccine is finally administered because only some plants can be consumed raw whereas others must be processed. With processing there is the potential that heat or pressure treatments could destroy the protein. Cereal crops are attractive species for expressing subunit vaccines because they can produce proteins in their seeds, which are stable for long storage periods. For animal vaccines, the plant could be chosen based on what is eaten as a major part of its diet, therefore eliminating the need to process the protein and the risk of destroying it as well (Daniell et al. 2001).

Regulation

In the United States, the production of proteins in plants to be used in the process of creating a pharmaceutical product will follow the same regulatory requirements that have already been established for non-plant produced pharmaceuticals. However, there are unique aspects associated with producing drugs in plant systems. These unique aspects will involve the Food and Drug Administration (FDA), the United States Department of Agriculture (USDA), and to a lesser extent the Environmental Protection Agency (EPA). The FDA regulates the testing, manufacturing and sale of pharmaceutical

products in the U.S., but the USDA will play a key role in the regulation of plant-made

pharmaceuticals. The USDA regulates the production and distribution of transgenic

plants, and is concerned with genetic containment and reducing the risk of gene transfer.

The agency is concerned with the transgenic plant's production process and also

considers risk management strategies for the production of these transgenic plants (Kirk

et al. 2005). Overall the EPA is charged with ensuring environmental safety, but the

USDA and FDA have the most regulatory responsibility associated with transgenic plants

for protein production to be applied to pharmaceutical applications. The FDA and USDA

regulate products that are registered, but also products under experimentation in the field,

laboratory, and greenhouse environments. The challenges that arise with an open

environment system and producing some of these pharmaceutical proteins in food-based

crops create potential new risks to be considered by each regulatory agency (Peterson and

Arntzen 2004).

Advantages of Plant-Based Pharmaceuticals

Production of antigens in transgenic plants began in 1990, but to date there are

still no vaccines for humans that have been produced using transgenic plants. The idea of

producing vaccines in plants came about to improve current methods of vaccine

production and advance the current immunization programs. Plants as protein factories

could reduce the cost of expanding production, potentially eliminating needles, and

producing heat stable vaccines that would also eliminate the cold chain processes, which

is a series of refrigeration steps en route from manufacturer to vaccination, currently in

place for delivering vaccines (Sala et al. 2003). Tobacco was the first plant transformed to produce *Streptococcus mutans* surface protein A and mice were successfully immunized with the plant material (Curtiss and Cardineau 1990). Curtiss and his group were also able to create transgenic alfalfa for expression of the enterotoxigenic *E. coli* heat labile enterotoxin B-subunit (LT-B), and successfully induced both mucosal and serum antibody responses (Curtiss, 1999). Following these demonstrations of expression of vaccine antigen in plants was the Norwalk virus capsid protein (Walmsley and Arntzen 2000), and the rabies virus glycoprotein expressed in transgenic tomatoes (McGarvey et al. 1995).

Not only would vaccine production in transgenic plants be helpful in developing countries, but it would also increase safety and reduce the cost of mass vaccination. Producing proteins in plants potentially is a safer alternative to mammalian and microbial cell cultures because they lack human pathogens, oncogenic DNA sequences, and endotoxins. The protein synthesis system pathway is also conserved between plants and animals and plants can fold and assemble recombinant human proteins efficiently. Bacterial systems sometimes fail to fold the proteins properly and then degrade or accumulate them as insoluble inclusion bodies (Twyman et al. 2003).

Drug companies often spend $500 million to $700 million and it takes approximately five to seven years to build new vaccine-fermentation facilities. By using genetically engineered plants costs could be reduced by 50 percent and development times could be shortened by 3-4 months (The Washington Times 2004). The costs of processing are reduced when the product is more concentrated in the starting material.

Expressing recombinant proteins in the seeds of transgenic plants allows high levels of

the protein of interest to accumulate in a small volume (Ma et al. 2003). Scalability is

relatively rapid when using plant-based systems versus cell systems because it is simply a

matter of planting more seeds and retrieving more seeds or tubers for protein extraction

and purification.

Plant-Cell Cultures for the Production of Therapeutic Proteins

Recombinant proteins can also be transformed into plant-cell cultures. Plant cell

suspension cultures produce recombinant proteins more rapidly than transgenic plants

because testing time to ensure transgene expression stability and testing against adverse

phenotypic changes in the host plant can take up to two years, whereas in plant cells the

development and testing is much shorter. Cell cultures also are contained in buildings

rather than produced in the open environment thereby, reducing the risk of contamination

to non-transgenic plants (Twyman et al. 2003). Tobacco plant cell cultures are grown by

disturbing the friable callus tissue that results in a homogeneous suspension of single

cells. The cultures are maintained in conventional microbial fermentation vats and the

system allows for fairly clean cultures (free of contamination) along with fairly

straightforward purification protocols (Ma et al. 2003).

Disadvantages of Plant-Based Pharmaceuticals

New technologies bring both risks and benefits and to protect the interest of

people it is necessary to assess the risks before introduction into society. The risks

associated with plants as protein factories include gene transfer through pollen dispersion to sexually compatible plants that are not genetically modified, and exposing the environment and its counterparts to antigens or selectable marker proteins. For instance, what happens to the insect or mammal that eats the plant? Risks are also associated with humans including oral tolerance, allergenic responses, inconsistent dosage, worker exposure and unintended exposure to antigens or selectable marker proteins in the food chain (Kirk et al. 2005).

Plants have differences in their post-translational modifications with respect to glycan-chain structure. Plants lack the terminal galactose and sialic acid residues that are found on many native human glycoproteins. Therefore there are changes in the glycosylation pathway that are required to produce proteins with typical human glycan structures in plants. One method to overcome this is to use purified human $\beta(1,4)$-glactosyltransferase and sialyltransferase enzymes for the in vitro modification of plant-derived recombinant proteins. Another method is to express the human $\beta(1,4)$-glactosyltransferase in transgenic plants to produce recombinant antibodies with galactose-extended glycans (Twyman et al. 2003).

Products, such as vaccines, are intended for healthy people and must be held to a high standard of safety assurance. Because vaccines are known to be among the safest medical interventions, many healthcare providers may not even entertain the idea that vaccination may be a potential cause of a post-vaccination deleterious event, thereby leading to substantial underreporting. (Ellenberg and Braun 2002).

Vaccine failure usually occurs because of inadequate storage or administration, or because of interference by related viruses. Approximately 10,000 reports per year are submitted to The Vaccine Adverse Event Reporting System (VAERS). VAERS is a passive surveillance system for monitoring the safety of vaccines in the U.S. It began in 1990 and is managed by the Centers for Disease Control (CDC) and the Food and Drug Administration (FDA). About 15% of the 10,000 reports describe a serious event, defined for regulatory purposes as an event resulting in death, life-threatening illness, hospitalization, prolongation of existing hospitalization, or permanent disability. (Ellenberg and Chen 1997). Vaccines are traditionally produced from the attenuated version of the virus itself and grown in cell cultures or hens' eggs.

After a vaccine is given, it is rare that a severe side effect will occur. More often there is only redness or soreness from the injection. People rarely experience allergenic reactions to a vaccine and since it does not occur very often it is difficult to calculate the risk of a vaccine's allergic potential. There is sometimes more risk associated with not receiving the vaccine than receiving it because the disease itself could be fatal (CDC 2005).

Mammalian and Bacterial Cell Culture Systems

Bovine cell culture systems or mammalian cell culture systems are the standard methods for producing glycosylated proteins (i.e. monoclonal antibodies). These culture systems may be able to offer properly folded and modified proteins, but the low yields per cost of production facility are a hindrance. Producing proteins in mammalian cell

cultures poses the risk of infecting the batch with human pathogens and bovine viral

diseases, such as bovine viral diarrhea, and also the degenerative central nervous system

disease, bovine spongiform encephalopathy (BSE) (Bioprotein Technologies 2005).

Microbial cell culture systems are also commonly used for the production of

proteins. They are very efficient and offer a low cost of production, however they are

limited to producing only simple non-glycosylated proteins because these do not require a

sophisticated folding process. The downstream processing after the protein is produced

in these systems is expensive. Scalability is difficult as well because it requires the

production of new facilities with fermentation vats and also hiring skilled laborers.

Objectives

The purpose of my project will be to assess specific ecological and human-health

risks from the new technology platform of plant-cell derived and whole-plant derived

pharmaceuticals and biologics. Quantitative human-health and ecological risk

assessments will be conducted to characterize the risks associated with growing

pharmaceutical proteins in crop-based plants. A comparative risk assessment will also be

conducted on a poultry vaccine that is produced in a plant cell culture as well as a risk

assessment on the conventional method of growing the virus for vaccine production in

bacterial or mammalian cell cultures.

I will use a formalized method of risk assessment for examination of hazards and

risk issues. Reasonable worst-case scenarios will be assumed for each protein and virus

being assessed and different exposure scenarios will be described and applied to each

protein and virus. The three proteins of interest that are currently produced in whole-plant systems (corn) include aprotinin, gastric lipase and *Escherichia coli* heat-labile enterotoxin B subunit (LT-B). The virus that is grown in tobacco plant cell cultures and extracted for vaccine production is Newcastle disease virus. Newcastle disease vaccine is used as an example of a plant-cell cultured vaccine to show the differences of risks when compared to whole-plant systems, and microbial and bacterial systems.

Risk Assessment

Uncertainties in the evolving regulatory environment and the need to evaluate the human-health and environmental risks of plant-based pharmaceuticals on a case-by-case basis necessitate using a robust, transparent science-based approach. Risk assessment is a formal discipline that objectively evaluates risk in which assumptions and uncertainties are clearly defined (NRC 1983). The process of risk assessment flows in a logical stepwise fashion that includes the following five steps: (1) problem formulation; (2) hazard identification; (3) dose-response relationships; (4) exposure assessment; and (5) risk characterization (Figure 1). Hazard and dose are considered in relation to exposure to determine risk or what additional information is needed to calculate or refine risk estimates (NRC 1983).
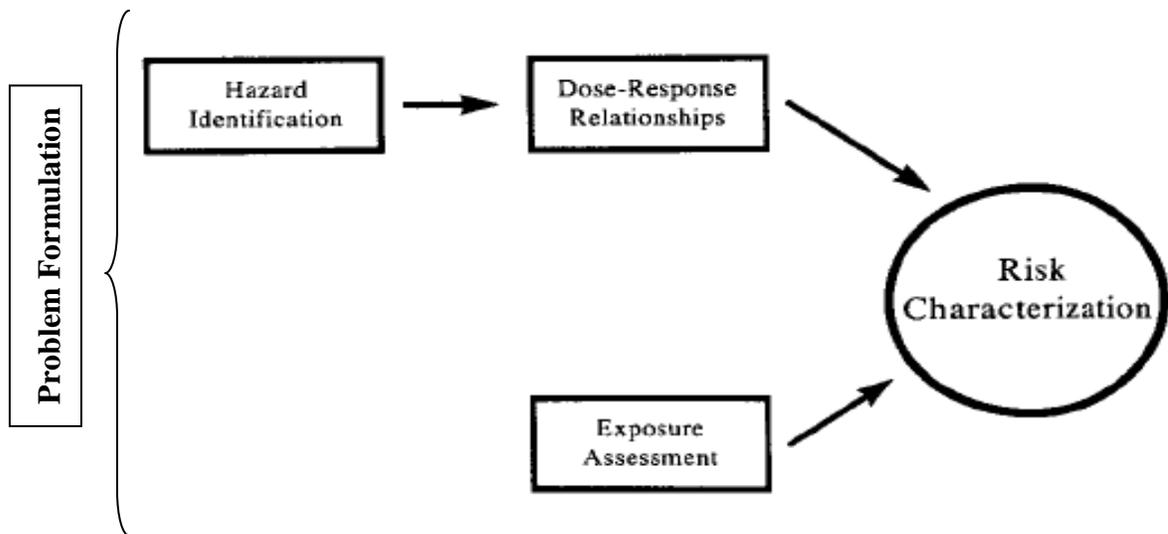
Figure 1. Risk Assessment Paradigm.

The problem formulation step sets the stage for the assessment establishing the

goals and the focus of the assessment. In the next step, the analysis phase, the effects and

exposures are characterized. The effects are any stressor that may affect the receptor and

the exposures are the interactions of the stressor with the receptor. These are defined as

reference dose, LD50's, NOEL's, and data assumptions based on time, place, and how

much of the stressor is present. The final step is risk characterization. This phase is the

consideration of the effects and exposures that were calculated previously to determine a

final risk. This step states the probability of the risk and also determines if more data is

needed to refine the risk assessment (Peterson and Hulting 2004).

The problem formulation step might ask questions regarding the harm or impact

the stressor or activity might have on the environment. There may be concern about

human health effects from the stressor, or the route of exposure it is taking. These

questions regarding a chemical or pesticide in the environment may be clearer and easily

answered but when trying to answer these questions regarding therapeutic proteins that are inserted into crops and grown in the open environment, the actual hazard may not be evident.

A risk assessment utilizes a tiered modeling approach beginning with a tier 1 assessment in which extremely conservative assumptions are used to screen out negligible risks. A tier 1 assessment is also deterministic, describing a single scenario, with high uncertainty and little data. However when moving up in the tiers the more refined the assessment becomes. In a tier 4 assessment (the highest) the uncertainties are addressed and the exposure scenarios are highly defined and often times multiple scenarios are used. Probabilistic models are often used, and assumptions are typically verified by monitoring techniques making higher tiered approaches more expensive to conduct. A higher tiered assessment typically means a closer-to-reality description of the hazard or stressor that was defined. Even though a tier 1 assessment may not give a complete understanding of the system it does help to provide information for regulators to make decisions and communicate to the public.

<center>Methodology</center>

Evaluations of the potential risks before a product is released into the environment to understand threats to food safety or the environment before they occur have yet to be used consistently to assess nonfood products derived from transgenic plants. The FDA drafted a guidance stating a zero tolerance for nonfood transgenic crops in the food supply (USDA 2002). It is unlikely that a zero tolerance will always be met; therefore a risk assessment in which environmental impact to surrogate species, and inadvertent

mixing in the food supply will be evaluated at levels above tolerable levels to assess the

risks as if these events did occur.

Tier 4

Tier 3   Refined Assessment

Tier 2

Tier 1

Conservative Assumptions
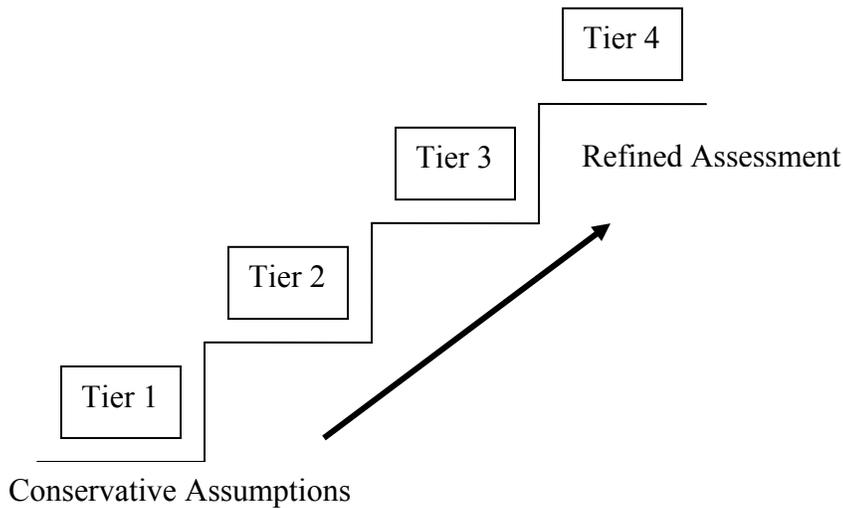
Figure 2. Tiered Approach to Risk Assessment.

Quantitative human health and ecological risk assessments will be conducted for

aprotinin, gastric lipase, and LT-B expressed in corn. A comparative risk assessment will

be conducted on the NDV vaccine. Effect and exposure scenarios will be modeled for

each system and each scenario will be based on a tiered approach in which inadvertent

exposure through ingestion will be examined to determine a risk characterization of the

plant-based pharmaceuticals.

The therapeutic proteins and vaccine we have chosen for this project are

significant because they have been expressed in whole-plant systems or in the case of

NDV it has been expressed in a plant-cell culture. By already being expressed in plant

systems it provides information for each therapeutic to conduct a risk assessment. Even

though some of the information will have to be assumed we have some starting points with these proteins to conduct a formalized risk assessment. The risk assessment will be useful for future proteins that have the potential for expression in plants and grown in cell cultures in a closed system or in the whole plant and grown in the open environment. It may provide a model to evaluate future plant-based proteins and assess them on a case-by base basis.

<div align="center">Human Dietary Risk Assessment</div>

Effect

Evaluation of acute ingestion toxicity endpoints will be obtained based on information from known toxicological endpoints. The expression of each therapeutic protein expressed in the maize kernels will be obtained through current literature sources and will be equated with the toxicity endpoint values to determine potential human and ecological acute risks from each pharmaceutical protein. The poultry vaccine expressed in plant-cell cultures will be evaluated qualitatively because quantitative information is not available to determine risk quotient values. The exposures will be compared to corresponding effect levels and to the risk quotients of aprotinin maize, gastric lipase maize, and Lt-B maize. The exposures for plant-cell derived NDV will be compared qualitatively to the exposures of conventionally derived NDV.

Exposure

The Food Commodity Intake Database (FCID) will be used with the Continuing Surveys of Food Intake by Individuals (CSFII) to determine the exposure and dietary

consumption of individuals consuming maize products. The FCID is a database that was developed by the USDA for use by the EPA and other organizations when conducting exposure components of dietary risk assessments (EPA 2005). The CSFII was created by the USDA and it measures the foods actually eaten by individuals (USDA 2002). The survey collects data that include demographics such as household size, income, race, age, sex, and where the food was purchased, how it was prepared, and where it was eaten. The Dietary Exposure Evaluation Model (DEEM) uses the information from the CSFII; therefore this project will use DEEM to estimate the inadvertent dietary intake of the therapeutic proteins in food (Kidwell et al. 2000). The goal of using this model is to determine the exposure of the population of concern and identify the variability in that exposure. The model includes an adjustment factor in which percentage of the crop (maize in this case) can be entered according to how much will reach non-transgenic maize though pollen flow.

Ecological Risk Assessment

The ecological risk assessment for aprotinin, gastric lipase, and LT-B will use surrogate species to determine dietary risk while employing the same exposure scenarios. The four species will include an ungulate species (deer), a rodent species (vole), an avian species (bobwhite quail), and a ruminant species (feeder and slaughter cattle). The species were chosen based on the ability to extrapolate effect data to these species and as a result successfully conduct an ecological risk assessment. These are all vertebrate species and information gained from this risk assessment will correlate nicely with the

human dietary risk assessment. Effect information is not currently available for insects, fish, and soil dwelling organisms (earthworms, microorganisms).

Poultry Risk Assessment

A comparative risk assessment for NDV will be conducted by examining the risks to chickens through a plant-cell derived method of obtaining a vaccine and through the conventional method of producing the vaccine in eggs. The assessment will focus on the requirements for veterinary biologics based on the Code of Federal Regulations (CFR) (CFR 2004). The CFR is concerned with the purity, potency, and efficacy of the biologic and the risk comparison can be made based on conventional purity, potency, and efficacy to the purity, potency and efficacy to plant-cell based vaccine production methods.

CHAPTER 2

A HUMAN DIETARY RISK ASSESSMENT

FOR PLANT-BASED PHARMACEUTICALS

Abstract

Genetic engineering has made it possible to use plants as factories for pharmaceutical protein production. Protein-based drugs are the fastest growing class of drugs for the treatment of disease in humans and other animals. However, the current method of producing proteins for pharmaceutical application is predicted to fall short in the years to come because of population growth and demographic trends. Maize is an attractive vehicle for cloned vaccine antigens and other pharmaceutical proteins because it is capable of being processed into several palatable forms, is inexpensive to maintain, and is also free of human and veterinary disease agents. This study characterized human-health risks using quantitative risk assessment techniques for three pharmaceutical proteins produced in field-grown maize. The risks associated with inadvertent consumption of pharmaceutical proteins in food as a result of production of those proteins in transgenic maize were characterized. The three proteins were aprotinin, gastric lipase, and *Escherichia coli* heat-labile enterotoxin B subunit (LT-B). The human dietary risks from the three proteins inadvertently occurring in food were evaluated using three different exposure scenarios so that potential risks could be compared. The three exposure scenarios ranged in conservatism to evaluate the range of risk between the proteins and scenarios. Risk quotients (RQ's) were calculated for all three scenarios to

integrate exposure and effect (toxicity). The dietary exposure values were determined probabilistically within DEEM. The human-health risk assessments revealed that the most conservative scenario produced higher RQ's than the other two scenarios. The dietary risks from Scenario 1 were at least 1000 times greater than Scenario 2 and at least 14,000 times greater than Scenario 3. This risk assessment revealed that human-health risks will vary dramatically and depend on factors such as the specific pharmaceutical protein, protein expression, and exposure scenarios. The assessment also reinforced the need for case-by-case assessments.

Introduction

Pharmaceuticals using genetic engineering have been made through protein expression in bacterial, fungal, and mammalian cell cultures. Biotechnology is currently evolving to produce more complex and diverse pharmaceutical proteins in plants. These pharmaceuticals are known as plant-made or plant-based pharmaceuticals.

Genetic engineering has made it possible to use plants as factories for pharmaceutical protein production. Plant-based pharmaceuticals are made by inserting a segment of DNA that encodes the protein of choice into the plant cells. The plants or the plant cells are essentially factories used to produce the desired proteins and are only grown for the purpose of pharmaceutical applications (Shama and Peterson 2004).

Many people suffer from infectious, inflammatory, and cardiovascular diseases— and these numbers are growing. Protein-based drugs are the fastest growing class of drugs for the treatment of these diseases in humans and other animals. However, the current method of producing proteins for pharmaceutical application is predicted to fall

short in the years to come because of population growth and demographic trends
(Giddings et al. 2000; Rogers 2003).

Maize is an attractive vehicle for cloned vaccine antigens and other
pharmaceutical proteins because it is capable of being processed into several palatable
forms. Maize-based antigens are also inexpensive to produce and scale up. The
distribution of the cloned antigen within the maize kernels is homogeneous, allowing for
a reproducible dose (Fischer and Emans 2000). Maize is also free of human and
veterinary disease agents. Maize-based vaccines would be well suited for developing
nations where refrigeration during storage and distribution is often difficult, and syringes
and other supplies for immunization are expensive and unsafe. The proof-of-concept for
transgenic plant vaccines has been demonstrated in animal models and extensive trials
are underway with promising results (Ma et al. 2003; Peterson and Arntzen 2004).

Plant-based pharmaceutical production has created public concerns about human-
health and ecological risks from exposure in the open environment and contamination of
the food supply (Peterson and Arntzen 2004; Freese 2005; Kirk et al. 2005). The
traditional method of vaccine production includes containment in facilities where cell
cultures are grown and not exposed to the open environment. With this new method of
growing transgenic crops expressing proteins for pharmaceutical production, those
proteins will be exposed to the environment. However, in a field there are special
containment practices that can be employed to reduce the risk of contamination from the
transgenic plants to non-transgenic plants. Risks pertaining to this method of producing

pharmaceutical proteins will need to be evaluated to ensure proper precautions are taken before cultivating these plants in the environment.

Pharmaceutical proteins have different structures, stabilities, and toxicities, and, because of this, regulatory guidelines should address each plant and protein combination on a case-by-case basis (Peterson and Arntzen 2004). "The route and frequency of administration should be as close as possible to that proposed for clinical use," but the clinical use of the protein may not result in the same toxicity of indirect exposure through adulterated food consumption (IHC 1997). By using a risk-based approach to address these concerns, and others, many of the implications can be resolved or managed by knowing where the risk occurs in the system.

Risks can be understood using the science-based paradigm of risk assessment. Risk assessment is a formal discipline that objectively evaluates risk, in which assumptions and uncertainties are clearly defined (NRC 1983). The process of risk assessment flows in a logical stepwise fashion. Hazard and dose are considered in relation to exposure to determine risk or what additional information is needed to calculate or refine risk estimates (NRC 1983).

The problem formulation step sets the stage for the assessment, establishing the goals and the focus of the assessment. In the next step, the analysis phase, the effects and exposures are characterized. Effects (hazard identification and dose-response relationships) are the ways in which a stressor (hazard) affects a receptor (e.g., human). Exposures are the interactions of the stressor with the receptor and depend on the frequency and degree of contact between the two. Effects typically are expressed as

quantified toxicity endpoints, such as reference doses, LD50's, or no-observed-effect-levels (NOEL's). Exposures are quantified based on considerations of time, place, and how much of the stressor is present. The final step is risk characterization. This phase is the consideration of the effects and exposures that were calculated previously to determine a final risk. This step states the probability of the risk and also determines if more data are needed to refine the risk assessment (Peterson and Hulting 2004).

A risk assessment utilizes a tiered modeling approach beginning with a tier-1 assessment in which extremely conservative assumptions are used to screen out negligible risks. A tier-1 assessment is also deterministic, describing a single scenario, with high uncertainty and few data. Even though a tier-1 assessment may not give a complete understanding of the system, it does help to provide information for regulators to make decisions and communicate to the public. When progressing to higher tiers, the risk assessment becomes more refined, meaning a more realistic estimate of hazard and exposure. Probabilistic models are often used in higher tiers and may be verified by monitoring techniques, which often causes these higher tiered approaches to be more expensive.

The objective of this study was to characterize human-health risks using quantitative risk assessment techniques for three pharmaceutical proteins produced in field-grown maize. In particular, human dietary risks from three proteins inadvertently occurring in food were evaluated using the same three exposure scenarios so that potential risks could be compared.

Materials and Methods

Problem Formulation

Quantitative dietary risk assessments were conducted to characterize the risks associated with inadvertent consumption of pharmaceutical proteins in food as a result of production of those proteins in transgenic maize. The three proteins were aprotinin, gastric lipase, and *Escherichia coli* heat-labile enterotoxin B subunit (LT-B). Three dietary exposure scenarios of varying conservatism were evaluated. Percentiles of dietary exposure and risk were determined for demographic groups such as toddlers, children, and seniors.

Aprotinin Effects Assessment

Aprotinin is a polyvalent protease inhibitor that has been in clinical use since the early 1960's. Manufactured and sold by Bayer as Trasylol®, aprotinin is supplied as a solution containing 10,000 kallikrein inactivation units per milliliter (KIU/ml), which equals 1.4 mg/ml.

Aprotinin has been shown to decrease protease activity when administered in conjunction with proteins and peptides. The coagulation activity of aprotinin led to the recommendation on the product label to not be used on normally clotting patients due to the risk of thrombosis, but recently this risk has been discounted. In rare first use cases, aprotinin has caused life threatening anaphylactic reactions, and this risk increases with re-exposure (Bayer Pharmaceutical Corporation 2003). Currently aprotinin is used

intravenously mainly in cardiac surgery for its beneficial effect on the reduction of the perioperative blood loss. It is also referred to as "bovine pancreatic trypsin inhibitor," which affects known serine proteases such as trypsin, chymotrypsin, plasmin, and kallikrein.

Aprotinin was traditionally extracted from bovine lungs and is used mainly for medicinal purposes, but also is used in laboratory research and development for controlling degradation of proteins. Some of the medicinal applications include minimization of blood loss during cardiac surgery to reduce the amount of transfusions needed. It can also be used to reduce blood loss in orthopedic surgeries as well as in pediatric patients undergoing cardiopulmonary bypass surgeries and organ transplantation. Following surgery, aprotinin has been observed to reduce systemic inflammation. Acute pancreatitis is a disease that has benefited from the effects of aprotinin by inhibiting the pancreas from producing enzymes.

Route of Therapeutic Administration and Dosage. Aprotinin is administered via IV (intravenous route) because it is not bio-available (inactive) after oral administration. To increase the clinical use of aprotinin and decrease the protease activity, oral dosage with other drugs is required. Soybean trypsin inhibitors and gel-forming polymers such as plycarbophil are drugs than can be given in conjunction with aprotinin to improve bioavailability (Rinsho 1998).

Stability. Aprotinin is quite stable due to its compact tertiary structure. Because of this structure it can withstand high temperatures, pH extremes, acids, alkalies, organic solvents, or proteolytic degradation (Roche Corporation 2003). Only thermolysin has

been found capable of degrading aprotinin after heating to 60-80 °C. Aprotinin is soluble in water and in aqueous buffers of low ionic strengths. The protein is highly basic and will adhere to dialysis tubing and gel filtration matrices (Sigma, Technical Bulletin). It has been tested against kunitz-trypsin inhibitor and against peptic digestion (pH 1.2) and found to be stable for greater than 60 minutes. Aprotinin can form stable complexes and block active sites of enzymes, but binding to the active site by most aprotinin-protease complexes can be reversed and dissociated at pH greater than 10 or less than 13 (Sigma, Technical Bulletin).

Respiratory and Skin Reactions. Respiratory effects or bronchospasms have been reported rarely with aprotinin as a result of IV injection. Hypersensitivity reactions by IV injection have been reported as skin rash, acute uritcaria, and anaphylaxis (Adamcakova-Dodd et al. 2004). Vucicevic and Suskovic (1997) reported a case in which a 24-year-old man received aprotinin therapy to control bleeding after a tonsillectomy and, two days after the surgery, he was readmitted and received a constant infusion of aprotinin at a 20 mg dose to stop capillary bleeding. Two hours after the infusion of aprotinin, the patient had hypotension and collapsed peripheral veins, and the aprotinin was discontinued. The man was discharged six days later.

The promoter for aprotinin is expressed in organs of maize other than grain (Adamcakova – Dodd et al. 2004). This could potentially mean a route of exposure to people via pollen or contact with other plant parts. Inhalation of pollen could potentially result in exposure of the nasal mucosa and upper airways with aprotinin. This route of exposure has not been studied (Adamcakova-Dodd et al. 2004).

Allergic Reactions. Allergic reactions to primary exposure are rare (Berierlein et al. 2000; Milano et al. 2002). However, adverse reactions after repeated applications, especially within a few weeks, have been reported. Life-threatening anaphylactic reactions increase by 5% of cases upon re-exposure (Bayer 2000). Patients with a history of allergic reactions to drugs may be at greater risk of developing a hypersensitivity or anaphylactic reaction upon exposure to aprotinin (Adamcakova-Dodd et al. 2004). Hypersensitivity reactions can range from skin eruptions, itching, dyspnea, nausea, and tachycardia to fatal anaphylactic shock with circulatory failure (Adamcakova-Dodd et al. 2004). Because of concerns over allergenicity, a test is recommended for all patients before aprotinin treatment. A 1.4 mg (10,000 KIU) test dose is administered intravenously at least 10 minutes before the loading dose. With patients who have already been exposed, an intravenous dose of H1-histamine antagonist (antihistamine) is recommended before the loading dose. For some patients with a high risk of bleeding that have been exposed to aprotinin, re-exposure to aprotinin may outweigh the risk of serious allergic reactions (Dietrich 1998).

Pregnancy. There has been no evidence of teratogenic or other embryotoxic effects of aprotinin treatment in animal studies (Bayer 2000). Only limited amounts of aprotinin penetrate the placental barrier and no studies are available on the passage of aprotinin into the mother's milk. Because aprotinin is not bio-available after oral administration, the drug would have no effect on the baby (Adamcakova-Dodd et al. 2004). Aprotinin should only be used in the first three months of pregnancy and only after careful risk/benefit assessment (Bayer 2000).

Urogenital Effects. Kidney toxicity has been observed in patients and in clinical trials (Bayer 2000). Acute renal dysfunction is among the more serious complications after cardiac surgery and causes the need for dialysis in patients, which is associated with a high mortality rate (Fauli et al. 2005). In a study of 60 patients undergoing coronary artery bypass grafting surgery with cardiopulmonary bypass (CPB), the patients were randomized into three groups: placebo, low-dose aprotinin, and high-dose aprotinin. The placebo group received 280 mg of saline, the low-dose group received 280 mg of aprotinin, and the high-dose group received a loading dose of 280 mg, a constant infusion dose of 70 mg/h, followed by 280 mg of a pump prime dose. The study showed that aprotinin caused a significant increase in α 1- microglobulin excretion but not in N-acetyl-β-D-glucosaminidase (β-NAG) excretion during CPB, which may be interpreted as a renal tubular overload without tubular damage (Fauli et al. 2005). The rate of acute kidney failure was 0.5% for aprotinin compared to 0.6% for placebo. The rate of tubular necrosis was 0.8% for aprotinin compared to 0.4% for placebo. Aprotinin showed a high affinity for renal tissue and pre-clinical studies have shown deposits of proteins in the phagosomes of the epithelial cells for the proximal renal tubules that are not metabolized or excreted until after the seventh day after intravenous administration of the drug. A study involving 4,374 patients (3,477 men and 897 women aged 18 years and older) undergoing revascularization, found that the use of aprotinin was associated with a doubling in the risk of renal failure, requiring dialysis among patients undergoing complex coronary-artery surgery (Mangano et al. (2006). Other findings of the regression analysis included an increased risk of death with the use of aprotinin for

cardiovascular events and cerebrovascular events. Mangano et al. (2006) also concluded that the use of the less expensive generic medications aminocaproic acid and tranexamic acid are safer alternatives.

Thromboembolism. Thromboembolism is the formation in a blood vessel of a clot (thrombus) that breaks loose and is carried by the blood stream to plug another vessel. The clot may plug a vessel in the lungs (pulmonary embolism), brain (stroke), gastrointestinal tract, kidneys, or leg. Thromboembolism is an important cause of morbidity and mortality, especially in adults. Treatment may involve anticoagulants (blood thinners), aspirin, or vasodilators (drugs that relax and widen vessels) (http://www.medicinenet.com).

Obstruction of blood vessels, or thromboembolism, can occur with aprotinin treatment. Early signs of blood vessel blockage were observed on the pulmonary artery catheter of 3 cardiac surgery patients who were receiving a high-dose of aprotinin (Anonymous 2001). The obstruction was observed 45-55 minutes after the catheter was inserted. A case of a 69 year old woman had a thrombotic event after cardiopulmonary bypass surgery and the event was attributed to aprotinin in which the woman had received a total dose of 6×10^6 KIU before and during the surgery (Anonymous 2001).

Cardiovascular effects. Tachycardia has been observed in some patients treated with intravenous aprotinin. Tachycardia is a rapid heartbeat initiated within the ventricles, characterized by three or more consecutive premature ventricular beats. Ventricular tachycardia is a potentially lethal disruption of normal heartbeat (arrhythmia) that may cause the heart to become unable to pump adequate blood through the body.

The heart rate may be 160 to 240 (normal is 60 to 100 beats per minute)

(http://www.nlm.nih.gov/medlineplus/ency/article/000187.htm). Mangano et al. (2006)

conducted a comparative risk assessment by assessing three agents commonly given to

patients undergoing revascularization. The three agents were aprotinin, aminocaproic

acid, and transexamic acid, and all were compared to using no agent during surgery. A

group of surgeons monitored 3,000 patients who had been administered the three agents

and 1,374 patients who had been given no agent at all.   It was found that aprotinin was

associated with an increased risk of death compared to the other two agents (2.8% vs.

1.3%, $P = 0.02$), cardiovascular events (20.4% vs. 13.2%, $P<0.001$), cerebrovascular

events (4.5% vs. 1.6%, $P<0.001$), and renal events (5.5% vs. 1.8%, $P<0.001$). Aprotinin

was associated with a 48 % increase in risk of myocardial infarction ($P<0.001$) and a

109% increase in the risk of heart failure ($P<0.001$) (Mangano et al. 2006). Sedrakyhan

et al. (2006) argued that the observational study conducted by Mangano et al. (2006)

contradicted the conclusions of the meta-analysis of 35 randomized trials—3,879

patients undergoing coronary-artery bypass grafting—whichshowed no increased risk of

myocardial infarction, renal failure, or stroke, an was in line with results of previous

meta-analysis. Mangano et al. (2006) reported cardiac, renal, and cerebral complications

to be almost double in frequency in patients treated with aprotinin as compared to

patients not receiving antifibrinolytics.

Pancreatic Disease from Ingestion of Protease Inhibitors. There are two classes

of protease inhibitors in plants. Serine protease inhibitors that inhibit proteases, trypsin

and chymotrypsin, and cysteine protease inhibitors that inhibit enzymes with a cysteine

in their active site. Both classes of protease inhibitors have been shown to have a growth retardant effect on insect pests and fungi. The protease inhibitors inhibit the digestive enzymes in the gut of insects. The proteases in the plant raise the question of any possible health hazards they pose to humans.

Animal experiments have shown that protease inhibitors decrease growth by interfering with protein digestion and lead to hypertrophy and hyperplasia of the pancreas. The pancreas reacts to this interference by enlargement and abnormal secretory activity. Aprotinin could potentially cause pancreatic disease in animals upon ingestion (SAP Report No. 2000-03B. 2000) because it is a serine protease inhibitor.

It is unknown if the same effects would be observed in humans after ingestion (SAP Report No. 2000-03B. 2000). However, introduction of soybean-derived serine protease inhibitor into the duodenum of humans caused a significant increase in the ability of the pancreas to secrete trypsin, chymotrypsin, and elastase (SAP Report No. 2000-03B. 2000). This demonstrates in humans that the pancreas responds negatively to the effects of a protease inhibitor (Adamcakova-Dodd et al. 2004).

A study conducted by Dlugosz et al (1988) evaluated the effects of aprotinin on tryptic activity by mixing a 1:1 ratio of duodenal juice samples from the pancreas with either saline solution or with aprotinin solution (25000 KIU/ml). The mixtures were incubated for 10 minutes at 37ºC, and kept on ice for 1 hour to simulate the fate of aprotinin infused introduodenally, and aspirated together with bile-pancreatic juice. The tryptic activity was inhibited from aprotinin by $91 \pm 2.6\%$ (Dlugosz et al. 1988).

In both insects and mammals, protease inhibitors may inhibit digestive enzymes and consequently stimulate over-secretion of these same enzymes in a negative feedback loop. In mammals, this leads to pancreatic disease. In insects, this triggers a third effect, depletion of essential amino acids, which may be the chief mechanism of toxicity. It is unknown in humans if it could deplete amino acids, causing nutritional deficiencies (Freese 2005).

Glycosylation. The carbohydrate sequence used in plant glycosylation is slightly different than that produced in mammals. In most cases this difference does not lead to any functional differences in the proteins, with one notable exception. Plants have differences in their post-translational modifications with respect to glycan-chain structure. Plants lack the terminal galactose and sialic acid residues that are found on many native human glycoproteins. Therefore, there are changes in the glycosylation pathway that are required to produce proteins with typical human glycan structures in plants. Aprotinin produced in transgenic maize seed is not glycosylated, and its structure-function characterization does not include glycosylation (Azzoni et al. 2002; ProdiGene 2004). The sequence of aprotinin was entered into a database to predict glycosylated residues. According to the results, the sequence may not contain a signal peptide. Proteins without signal peptides are unlikely to be exposed to the O-glycosylation machinery and thus may not be glycosylated (*in vivo*), even though they contain potential motifs. So far there is no evidence that such differences cause adverse reactions in human patients (Ma et al. 2003).

Effects on Pediatric Patients. The effects of aprotinin on pediatric patients undergoing cardiopulmonary bypass surgeries are controversial (Adamcakova-Dodd et al. 2004). Costello et al. (2003) administered aprotinin to children six months of age or younger and showed that aprotinin reduced operative closure time and blood transfusion and reported no observed allergic reactions. Jaquiss et al. (2002) reported no long-term ill effects of aprotinin reactions in children. It was reported that the risk for hypersensitivity reactions to aprotinin is low in young children undergoing cardiothoracic surgery, even with multiple exposures to the medication. Reactions are more likely with re-exposure, and risk increases with multiple exposure (Adamcakova-Dodd et al. 2004). Aprotinin seems to be beneficial in pediatric organ transplantation, and repeat use of aprotinin appears to be safe and does reduce blood loss in re-transplantation patients, but allergy to aprotinin is a concern. However, according to the Trasylol label, safety and effectiveness in pediatric patients have not been established (Bayer 2000).

Aprotinin Toxic Endpoints. The toxicity endpoints for the human health risk assessment for aprotinin were based on the dose regime that is administered to patients undergoing cardiopulmonary bypass surgery. The loading dose for patients was chosen as the toxic endpoint over the dog NOEL determined by Trautschold et al. (1976), because the dog NOEL did not seem appropriate for a human toxic endpoint. Adverse events have been observed in patients who received the loading dose during surgery, and it is a more conservative value than the dog NOEL. The loading dose was then divided

by the body weight of an average adult male (70 kg), which gave the endpoint value (280

mg aprotinin ÷ 70 kg = 4 mg/kg body weight (BW)).

Gastric Lipase Effects Assessment

Gastric lipase is a protein that was developed as a pharmaceutical to aid in the

treatment of exocrine pancreatic insufficiency (EPI), which is an incapacity in the human

body to send digestive enzymes to the gut to assimilate food particles (Basque and

Ménard, 1999). The absence of lipase in the body does not allow digestion of food

lipids, which leads to a condition known as steatorrhea, which means there are excess

fatty deposits in feces. This disease (EPI) mainly affects cystic fibrosis patients. Lipases

are produced naturally in many animals, plants and microbes, and have been consumed

for many years without any adverse effects (Coenen et al. 1997).

Acute Oral Toxicity. Two groups of five female and five male rats in each group

were orally administered once via gavage with recombinant lipase expressed in

Aspergillus oryzae. The rats weighed 70-76 g and were given 5 g/kg BW of lipase. The

rats were observed for 14 days and then necropsied. All survived the 14-day treatment

and showed no clinical signs of toxicity. Therefore, the LD_{50} and NOEL exceeded 5 g/kg

BW (Stavnsbjerg 1988; Greenough et al. 1996).

Flood and Kundo (2003) studied groups of 12 male and 12 female Sprague-

Dawley SPF (specified pathogen free) rats at six weeks of age that were dosed with

Lipase D (activity 3,170,000 U/g, defined as the activity of one gram of pure enzyme

protein) by gavage at levels of 0, 500, 1,000, and 2,000 mg/kg BW/day for 13 weeks

with a dose given everyday. Additional groups of six males and six females received 0

or 2,000 mg/kg BW/day during the treatment period (13 weeks) and a four-week

recovery period. Animals were observed three times daily and body weights and food

consumption were recorded twice weekly. No adverse clinical signs or ophthalmologic

abnormalities or deaths were observed, and food consumption and body weights did not

differ significantly from the controls. There were no ocular changes or changes in

hematology parameters. Even though there was reduced urinary pH and

histopathological effects observed in the male rats at the highest dose of 2000 mg/kg BW

they were not considered to be toxicologically significant, just an increase in metabolites.

The NOEL for males was 1,000 mg/kg BW, and the NOEL for females was 2,000 mg/kg

BW (Flood and Kundo 2003).

Acute Inhalation Toxicity. (McDonald, 1988). Five male and five female

Sprague-Dawley rats weighing 115-142 g were confined in a nose-only inhalation

chamber and exposed for four-hours to an atmospheric concentration of 0.74 +/- 0.1

mg/liter. This is the highest concentration that was maintained over the four-hour period.

The inhalation median lethal concentration (LC_{50}) was not demonstrated in this limit test

other than an indication that the value exceeded 0.74 mg/liter (Greenough et al. 1996).

Subchronic Oral Toxicity Studies. McDonald and Parkinson (1988) dosed 10

male and 10 female Sprague-Dawley rats once per day by gavage at levels of 0, 0.2, 2, or

10 g/kg BW for two weeks. Perry et al. (1989) examined Sprague-Dawley rats over a

period of 13 weeks. Twenty male and 20 female rats were dosed a volume of 10 ml/kg

BW to give dose levels of 0, 0.2, 1, and 5 g/kg BW. No treatment-related clinical signs

were seen in either study after measuring organ weights, gross pathological examination, and microscopic examination of a comprehensive list of tissues in control and high-dose animals (Greenough et al. 1996).

Skin Irritation. The backs of 12 albino rabbits were shaved, and 0.5 g of test material was introduced under gauze. The test materials (batch C and batch A) used was from batches of *Humicola lanuginose* lipase expressed in *Aspergillus oryzae*. The skin was abraded in one area and intact in the other. Patches were secured onto the test sites for four hours and then removed with water after the four-hour period and skin reactions were evaluated at 1, 24, 48, and 72 hours after removal. A primary irritation index from test batch C was classified as a mild irritant, and a non-irritant was classified from test batch A (Greenough et al. 1996).

Eye Irritation. Test batch C was used for an eye irritation study in which three albino rabbits were administered 0.1 ml of test material into the conjuctival sac of the left eye. The eyes were evaluated at 1, 24, 48, and 72 hours. No corneal or iris reactions were observed (Greenough et al. 1996).

Skin Sensitization: Delayed Contact Hypersensitivity. Berg (1988) used test batch C to test for induction and challenge procedures on groups of 20 guinea pigs to determine whether sensitization occurred. The induction test required injection of 20 guinea pigs four times with 0.1 ml of test material at a concentration of 500 LU/g, or 500 lipase units per gram. Ten guinea pigs were injected with 0.9% sterile saline as negative controls. They were also challenged epicutaneously 13 days later with a six-hour patch

containing the test material (30,000 LU/g). A week later all the animals were challenged intradermally in the flank with 0.1 ml test material (200 LU/g). One week after the intradermal challenge they were all challenged epicutaneously to confirm the first epicutaneous challenge. Sensitization was not observed (Greenough et al. 1996).

Gene Mutation. Pederson (1988) used test batch A to examine mutagenic activity by using various bacterial strains. Bacteria were exposed to five doses of test material in a phosphate buffered nutrient broth for 3 hr (0.1 to 10 mg/ml incubation mixture at half-log intervals). The test was conducted in the presence and absence of metabolic activation by a liver preparation from male rats. It was concluded that there was no mutagenic activity in the presence or absence or metabolic activation (Greenough et al. 1996).

Chromosome Aberrations. Marshall (1988) used test batch A to observe mitotic inhibition at specific dose levels, in an *in vitro* assay of human lymphocyte cultures from a male and a female donor. Treatments were done on the presence and absence of metabolic activation by a rat liver post-mitochondrial fraction (S-9) from Aroclor-1254 induced animals. The test material was unable to induce chromosome aberrations in human lymphocytes when tested up to 5,000 μg/ml in either the absence or presence of S-9. There was no evidence of treatment-related mitotic inhibition at any of the analyzed dose levels (Greenough et al. 1996).

Toxicity of Lipase from Genetically modified *Aspergillus oryzae*. The enzyme lipase is currently permitted for use as a processing aid, including when sourced from a

genetically manipulated strain of *A. oryzae*. To approve the use of lipase from a genetically modified microorganism involves the use of two organisms: *A. oryzae* (the source organism) and *F. oxysporum* (the donor organism). *A. oryzae* is currently listed in Standard 1.3.3 Processing Aids as a microorganism permitted for use in the production of certain enzymes, and has a history of safe use. The genetic modification process involves the transfer of the lipase gene from *F. oxysporum* to *A. oryzae.* The recombinant organism was found to be stable during production fermentations. Southern blotting technique was used to investigate the stability of the integration of the lipase gene after large-scale fermentation, and found that the DNA was stably integrated into the host genome.

Three toxicological studies were done to test the safety of lipase from the source organism (*A. oryzae*) carrying the gene from *F. oxysporum* (ANZFA 2002). The studies consist of a 13-week oral toxicity study in rats, a bacterial mutagenicity assay (Ames test), and a human lymphocyte cytogenetic assay. The 13- week oral toxicity study administered lipase by gavage to three groups of rats (10/sex/group) at doses of 0.083, 0.249, and 0.830 g TOS (total organic substance)/kg/day using a constant dose volume of 10 ml/kg BW/day for 13 weeks. The control group received tap water. The daily treatment with test substance at concentrations of up to 0.830 g TOS/kg/day for 13 weeks resulted in no treatment related effects. Therefore, the NOEL for lipase is 0.830 g TOS/kg/day, which was the highest dose in the study.

A test for mutagenic activity examined strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and *Escherichia coli* WP2uvrA. A liquid culture

assay was applied, and bacteria were exposed to six doses of test substance in a phosphate buffered broth for three hours with 5 mg/ml as the highest concentration. After incubation, the number of revertants to prototrophy and viable cells was estimated. The study comprising *E. coli* used the direct plate incorporation assay. The test was carried out both in the presence and absence of metabolic activation, with 5 mg/plate as the highest dose level, followed by bi-sections between doses. No dose-related or reproducible increases in revertants to prototrophy were obtained with any of the bacterial strains exposed to lipase. The study also confirmed that lipase did not exhibit any mutagenic activity under the conditions of the tests. The third study, concerned with the potential of lipase to damage the chromosomal structure in human lymphocytes, tested human lymphocyte cultures from a female donor. The first experiment in the absence and presence of metabolic activation in the form of S-9 lasted 3 hours with 17 hours recovery prior to harvest. The highest concentration chosen for analysis was 5,000 μg/ml and induced at approximately 12% and 42% mitotic inhibition in the absence and presence of S-9, respectively.

The second experiment was continuous for 20 hours in the absence and presence of S-9. Treatment in the presence of S-9 was for 3 hours, followed by a 17-hour recovery period. The concentrations for this experiment, 5000 μg/ml and 1638 μg/ml, induced approximately 0% and 53% mitotic inhibition in the absence and presence of S-9, respectively. The treatment did not produce biologically-or statistically-significant increases in the frequency of aberrant chromosomes at any concentration tested when compared to control values. Positive controls (4-nitroquinoline-1-oxide and

cyclophosphamide) gave the expected increases in frequency of aberrant metaphases.

The enzyme activity was found to be 4,000 LU/g (defined as the activity of one gram of

pure enzyme protein), and the total organic substance (TOS) content was 3%. The

enzyme preparation complies with the JECFA (Joint Expert Committee on Food

Additives) specifications, and the enzyme preparation causes no mutagenic or cytogenic

effects in *in vitro* studies. The NOEL from sub-chronic rat feeding studies was 0.830 g

TOS/kg/day (ANZFA 2002).

Because the genetic modifications were well characterized, well-known plasmids

were used for the vector constructs, and the introduced genetic material did not encode

and express any toxic substances, it was concluded that the use of this genetically

modified lipase as a processing aid in food would pose no significant risk to human

health (ANZFA. 2002).

Toxicity of Lipase Derived from *Rhizopus oryzae*. A lipase enzyme from

Rhizopus oryzae produced by fermentation was subjected to a series of toxicological

tests to document the safety for use as a food additive. The enzyme was examined for

acute, subacute, and subchronic oral toxicity, as well as mutagenic potential. Lipase

enzyme from *R. oryzae* has been consumed for many years as a digestive aid without

adverse effects (Coenen et al. 1997).

Acute Oral Toxicity. Five male and five female rats were given a single oral dose

by gavage of the lipase enzyme in the tox-batch at 5,000 mg/kg BW and were observed

for 14 days. The rats were killed and autopsied, and it was found that there was no

mortality and no clinical signs during the study. The LD_{50} of the tox-batch in rats of either sex was established as exceeding 5000 mg/kg BW (Coenen et al. 1997).

Subchronic 14-day Oral Toxicity. A 14-day study with four groups of five male and five female young SPF (specified pathogen free) rats were given the lipase enzyme in the tox-batch in purified water by oral gavage at doses of 0, 20, 100, 500, and 1,000 mg/kg BW/day. There were no changes in clinical appearance, body weight gain, food consumption, efficiency of food utilization and organ weight. Only minor changes in two males receiving 1000 mg/kg BW/day in their stomach walls were observed. The stomach walls were thicker when the rats were autopsied (Coenen et al. 1997).

Subchronic 90-day Oral Toxicity. The lipase enzyme in the tox-batch was administered to SPF-free rats by oral gavage. There were four groups with 20 males and 20 females. The doses were given with purified water, and administered daily. The dose concentrations were 0, 50, 200, and 1,000 mg/kg BW/day. There were no changes in body weight, food consumption, food conversion efficiency, opthalmoscopic examination, macroscopic examination, organ weights and microscopic examination. The NOAEL of the tox-batch in this study was 1,000 mg/kg BW/day (Coenen et al. 1997).

Lipase capsules (Pancrease brand of Pancrelipase). Pancrease capsules are a pancreatic enzyme supplement for oral administration. Pancrelipase is the active ingredient in the capsule and is harvested by extraction from the pancreas of the hog.

The lipase enzyme concentration in one capsule is 4,500 U.S.P. units (United States Pharmacopia unit) (equal to an international unit IU).

Teratogenic Effects. Studies have been conducted in rats and rabbits at doses 0.44 times and 0.35 times the maximum daily human dose, respectively, and have revealed no evidence of impaired fertility or harm to the fetus. There are no adequate and well-controlled studies in pregnant women. The drug should be used during pregnancy only if needed (McNeill Pharmaceutical 1992).

Nursing Mothers. Pancreatic enzymes act locally in the gastrointestinal tract and are not likely to be systemically absorbed. Some of the constituent amino and nucleic acids are likely to be absorbed along with dietary proteins. Thus, there is a possibility of the protein being in the breast milk (McNeill Pharmaceutical 1992).

Pediatric Use. Colonic strictures, in children with cystic fibrosis, have been associated with doses above the recommended dosing range. Patients currently receiving doses >2,500 lipase units/kg/meal or 4,000 lipase units/gm fat/day should be re-evaluated and the dosage immediately decreased to the lowest effective clinical dose, as assessed by three-day fecal fat excretion (McNeill Pharmaceutical 1992).

Adverse reactions. The most frequently reported adverse events resulting from the post-marketing experience with Pancrease (gastric lipase) were gastrointestinal and included diarrhea, abdominal pain, intestinal obstruction, vomiting, flatulence, nausea, constipation, melena, and perianal irritation. Other adverse reactions included weight decrease and pain and hyperuricemia and hyperuricosuria with non-coated formulations.

Some cases of fibrosing colonopathy have been reporting in cystic fibrosis patients

(McNeill Pharmaceuticals 1992).

Dosage. Dosage should be based on the individual and determined by the degree

of steatorrhea and fat content of the diet. The lowest possible dose should be given first

and then gradually increased until the desired level of steatorrhea is obtained. Children

less than four years of age can begin with a dosage of 1,000 U.S.P. lipase units/kg/meal

to a maximum of 2,500 lipase units/kg/meal, and children greater than four years old can

begin with a dosage of 400 U.S.P. lipase units/kg/meal to a maximum of 2,500 lipase

units/kg/meal. Doses are higher in infants because on average infants ingest five grams

of fat per kilogram of body weight per day, whereas adults ingest approximately two

grams of fat per kilogram of body weight per day. There have been no reports of acute

over dosage (McNeill Pharmaceuticals 1992).

Merispase Therapeutic Protein Produced by Meristem Therapeutics.

Clinical Trial of Recombinant Mammalian Gastric Lipase. The first clinical trial

conducted in healthy patients and in adult cystic fibrosis patients showed that maize-

derived gastric lipase was well-tolerated and that it resulted in improved assimilation of

lipids when combined with high doses of porcine pancreatic extracts. Meristem's gastric

lipase is currently in phase II clinical trials to define the dosage of lipase necessary to

substitute porcine pancreatic extracts completely. Meristem reported up to 5,000 mg/kg

BW acute toxicology in mice and rats. For dogs and monkeys, the dose was up to 2,500

mg/kg BW. A single IV dose in rats was reported to be up to 450 mg/kg/day. A four-

week toxicology study on gastric lipase reported rats could receive up to 500 mg/kg/day and micro pigs could receive up to 150 mg/kg/day. The expected active dose in cystic fibrosis patients is 10 mg/kg BW/day. There were no observed allergenic effects in the animals and humans after these studies (Meristem Therapeutics – risk assessment symposium April 2004).

Gastric Lipase Toxic Endpoints. The ingestion NOEL for gastric lipase is 1000 mg/kg BW in rats (Coenen et al. 1997). Lipases are produced naturally in many animals, plants and microbes, and the rat NOEL is the only known NOEL for this protein (Coenen et al. 1997). Because of its ubiquitous nature, this known NOEL may be extrapolated for a human dietary risk. Therefore, the toxicity endpoints were based on this value.

E. coli Heat-labile Enterotoxin B Subunit Effects Assessment

Escherichia coli heat-labile enterotoxin (LT-B) is made up of five "B" subunits and one "A" subunit. The "A" subunit is an active protein that causes adverse effects in the small intestine by entering the epithelial cells and causes water loss from the cells. The "B" subunit is harmless by itself but will provoke an immune response to the entire enterotoxin. LT-B is a non-toxic subunit that is part of the heat-labile toxin produced by enterotoxigenic strains of *E. coli*, which is the leading cause of diarrhea in developing countries.   LT is an 84-kilodalton oligomeric protein composed of two major noncovalently linked immunologically distinct peptides called LT-A and LT-B. LT-B is a 55-kilodalton homopentamer of 11.6 kDa peptides responsible for binding of the toxin

to the host-cell receptor galactosyl-N-acetylgalactosaminyl-sialyl galactosyl glucosyl

ceramide, which is found on the surface of eukaryotic cells (Chikwamba et al. 2002).

Route of Therapeutic Administration and Dosage. The antigen, *E. coli* heat-labile

enterotoxin B sub-unit (LT-B), induces an immune response when orally administered

and enhances immune responses to conjugated and co-administered antigens. LT-B also

has potential to be used as an oral vaccine against *E. coli* induced diarrhea. A human

clinical trial was designed to demonstrate that transgenic potato could induce an immune

response in humans. The trial involved 14 healthy adults, and all of the adults ate a dose

of raw potato. Three subjects were given ordinary potatoes, and the other eleven subjects

were given a potato expressing the LT-B antigen. Ten out of the eleven who ate

transgenic potatoes had a four-fold increase in the amount of antibodies to *E. coli* in their

blood. Six out of the eleven subjects showed a four-fold increase in the amount of

mucosal antibodies. A dose is equal to one raw potato, and each subject ate a total of

three doses (potatoes) in a four week period (Iowa State University 2003).

Stability. Transgenic corn expressing LT-B is stable to high temperatures that are

typical in food processing, especially with the antigen expressed in the kernel (Streatfield

et al., 2002). However, LT-B is degraded in simulated gastric fluids in less than five

minutes (Chikwamba et al. 2003).

Equivalence. For safety reasons, it is important that the antigen expressed in

plants is equivalent to the antigen derived from the original source. For corn expressed

LT-B, established equivalence to the microbial forms of LT-B in terms of activity and

physical characteristics is important as well as following the guidelines of the United States Food and Drug Administration (FDA) of establishing equivalence of the transformed host plant as part of the manufacturing process (Chikwamba et al. 2002; Lamphear et al. 2002 USFDA 2003). Maize expressed LT-B has been shown to be equivalent to the microbial forms in terms of activity and physical characteristics (Chikwamba et al. 2002; Lamphear et al. 2002).

Glycosylation. Post-translational glycosylation for LT-B expressed in maize has not been reported (Wolt et al.2006).

Oral Feeding Study. Feeding experiments in mice showed that transgenic corn meal expressing LT-B was capable of inducing strong serum and mucosal antibodies and protected mice from the *E. coli* labile toxin (Streatfield et al. 2001; Chikwamba et al. 2002). Chikwamba et al. (2002) showed that four doses of 10 μg LT-B/g of corn meal pellet were adequate to induce an immune response in mice. Using the gamma zein promoter, LT-B levels of up to 350 μg/g of dry kernel tissue were attained. The expression level was more than required to induce a protective immune response in mice. Mason et al. (1998) suggested that up to 1.1 mg would be required to induce a protective immune response in humans, and this dosage requirement could be met in 3 grams of dry maize meal from P77-7 R3 kernels. Lamphear et al. (2002) further investigated the immunogenicity of LT-B transgenic maize by examining defatted germ fraction in which the concentration of antigen is increased over whole kernels. Lamphear et al. (2002) also delivered the antigen to mice over a wider range of doses and included very small doses. The mice were fed defatted LT-B maize germ meal or defatted wild type maize germ

meal (negative control), and serum and fecal samples were collected and analyzed. They found serum IgG responses after the first dose and after the second dose for the lowest dose levels given to the mice. The serum IgG response increased throughout the study with continued doses. All the mice (10 out of 10) fed 3.3 or 33 μg of LT-B and the mice fed 0.33 μg responded (8 out of 10) with LT-B specific serum IgG assay values exceeding twice the average value for pre-immune serum. These data show that even 0.33 μg of LT-B antigen is sufficient to give a serum IgG response. The amount of maize material fed in this case was only 0.7 mg.

Transgenic maize expressing 1 mg of *E. coli* LT-B was fed to adult volunteers in three doses, each consisting of 2.1 g of plant material. Seven of the nine volunteers developed increases in both serum IgG anti-LT and numbers of specific antibody secreting cells after vaccination. Four of nine volunteers also developed stool IgA. This showed the LT-B in the plant material was an effective agent for an oral vaccine antigen (Tacket et al. 2004).

Tacket et al. (1998) showed no adverse effects in a human clinical trial in which the subjects ingested up to 1.1 mg LT-B (0.016 mg/kg BW for a mean adult body weight of 70 kg) from transgenic potato.

E. coli heat-labile enterotoxin B subunit and four other antigens have been successfully expressed in vegetables. Animal studies have been conducted for transgenic potatoes containing LT-B, Cholera toxin B subunit (CT-B), and the Norwalk virus capsid protein (Iowa State University 2003). The "B" subunit of *E.coli* was the protein expressed in potatoes. The transgenic potatoes were fed to mice and the mice were

exposed to the entire heat-labile enterotoxin. The mice that consumed the transgenic potatoes showed less fluid secretion in their intestines than the mice that had not eaten the transformed potatoes (Iowa State University 2003).

The interaction of LT-B with the immune system is not presently well understood. Ongoing research is seeking 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, it is necessary to be cautious when considering unintended occurrence of LT-B in food (Wolt et al. 2006).

Human Clinical Trial. The "B" subunit of *E. coli* heat-labile enterotoxin has been tested in human clinical trials to show that the transgenic potatoes could induce an immune response in humans (Iowa State University 2003). Fourteen healthy adults were chosen for the trial and ate a dose (dose not reported) of raw potato at the beginning of the study, and as the study progressed they consumed two more doses. Three of the individuals were given non-transgenic potato and the other 11 were given the transgenic potato expressing the LT-B antigen. The results showed that 10 of the 11 individuals had a four-fold increase in the amount of antibodies to *E. coli* in their blood, and six of the 11 had a four-fold increase in the amount of mucosal antibodies. There were no serious side effects reported from this trial.

Allergic Reactions. Homology with a known allergen is an indicator of a potential allergen for a transgenic protein. A transgenic maize variety expressing LT-B was screened against a database of known protein allergens and showed no matches for

35% or greater homology over an 80 amino acid window and no match of any eight contiguous amino acids (Gendel 1998; FAO 2001).

E. coli Heat-Labile Enterotoxin B Subunit Toxic Endpoints. In a clinical trial in which humans ingested up to 1.1 mg LT-B from transgenic potato, no adverse effects were identified (Tacket et al. 1998). The NOEL, with an average adult male human body weight of 70 kg, was reported as 0.016 mg/kg BW (Wolt et al. 2006). Therefore, this endpoint (NOEL) will be used for the human dietary risk assessment.

Exposure Assessment

Aprotinin Expression in Maize. The company, ProdiGene, began commercial scale-up of aprotinin in field-grown maize in [2003 and 2004]. Aprotinin traditionally has been extracted from bovine lungs. ProdiGene's recombinant aprotinin is equivalent to bovine aprotinin (ProdiGene 2002). Zhong et al. (1999) found that aprotinin was at a higher concentration in maize embryonic tissue than in endosperm tissue. Azzoni et al. (2002) reported the expression level of aprotinin produced from transgenic maize seed to be 0.17%; Zhong et al. (1999) observed an expression level of 0.1%.

Gastric Lipase Expression in Maize. The company, Meristem Therapeuticas, is developing a recombinant mammalian gastric lipase. Recombinant gastric lipase is grown in maize and then purified and extracted and used in pre-clinical studies. The mammalian lipase from porcine tissue was selected because it is naturally resistant to digestion by stomach acids and maintains a high enzymatic activity after passage through the stomach. The expression level is approximately 1 mg/g kernel and there is no expression in other

plant organs. The expression was observed to be stable over 11 generations (Meristem Therapeutics 2006).

LT-B Expression in Maize. Chikwamba et al. (2002) produced the B sub-unit of the enterotoxin *E.coli* heat-labile enterotoxin in transgenic maize seed. In their study, the LT-B gene was regulated by a 27-kDa gamma zein promoter, a seed-specific promoter and no LT-B expression was detected in callus tissues. For analysis of the LT-B protein levels in R1 seed, proteins from 20 kernels per ear representing each event were extracted separately and assayed for LT-B expression by ELISA. Data from all LT-B positive kernels in each ear were pooled to determine the mean and standard deviation for each ear. Levels of LT-B accumulation in P77 (one ear per event) and P112 (two ears per event) were shown. Of 19 LT-B expression P77 events, 11 had LT-B protein levels higher than 0.01% of total aqueous-extractable protein (TAEP). Two events (P77-2 and P77-3) had LT-B levels of up to 0.07%. P112 transgenic events showed the highest level of LT-B accumulation in R1 seeds. P112 events carry the construct pRC4-1, in which the endoplasmic reticulum-retention signal sequence SEKDEL was included in the C-terminus of LT-B gene. Inclusion of the SEKDEL motif in the LT-B gene under the regulation of the seed-specific promoter greatly enhanced the LT-B level in P112 events. Compared to transgenic maize plants carrying CaMV 35S promoter/LT-B constructs (P51 and P65), plants carrying zein promoter/LT-B constructs (P77 and P112) were more vigorous in overall performance.

Chikwamba et al. (2003) showed that LT-B protein was detected internally and externally in starch granules of maize. The strong association between the starch

granules and the protein gives an effective co-purification of the antigen in the processing of the starch fraction of the corn kernels, thermostability, and resistance to peptic degradation in simulated digestion fluids (Chikwamba et al. 2003). The strong association would help to understand and monitor for any chance of inadvertent occurrence, whereas the thermostability and resistance to peptic degradation indicates the antigen would be stable after ingestion and might be a concern if the LT-B maize is inadvertently mixed with food (Chikwamba et al. 2003).

Maize-derived LT-B has been extracted to reveal kernel expression levels of 9.2% of total soluble protein, and 3.7% of total soluble protein has been achieved by using seed specific promoters. As much as 350 μg/g LT-B could be expressed in kernels with the seed specific promoters (Chikwamba et al. 2002).

Protein Expression Assumptions for this Risk Assessment. Information on expression levels and protein distribution within various parts of the plant is needed to determine potential dietary exposure. Protein expression levels are not known for all of the proteins, but the expression of LT-B is typically 35-50 μg/g of dry seeds (about 4-5 kernels). Improvements in transformation and translation will be made over time and likely increase expression levels (Wolt et al. 2006). Therefore, for this human-health risk assessment, 100 mg/kg (ppm) and 1000 mg/kg (ppm) are used as point-estimate expression levels for all three proteins. Expression of 100 mg/kg represents what is currently being achieved, and 1000 mg/kg represents the potential protein expression in the near future.

Human Dietary Exposure Scenarios

Scenario 1. The first, and most conservative, exposure scenario for this risk

assessment assumed that the transgenic maize expressing the therapeutic protein was

harvested and accidentally taken to a specialty food processing facility. The specialty

food processing facility makes tortilla chips, and the transgenic maize was made directly

into the tortilla chips with no dilution from non-transgenic maize. During the food

preparation process, the protein was not denatured. The tortilla chips were then eaten by

individuals in a variety of age ranges. The expression level of the proteins was calculated

by multiplying the weight of a bag of Tostitos® tortilla chips (0.3827 kg) by each

expression level (100 ppm and 1000 ppm). The exposure was calculated by multiplying

the estimated protein expression levels by the consumption (assuming consumption of

the entire bag of chips in one day) and then dividing that product by the appropriate age-

specific body weights (Table 1). This exposure scenario was similar to Wolt et al. (2006)

in that the transgenic maize was harvested and made directly into tortilla chips with no

loss of protein function. However, their scenario assumed a dilution with non-transgenic

maize. Also, this risk assessment was conducted on a variety of demographic groups

rather than just a high-end consumer group.

Scenario 2 . The second exposure scenario assumed that there was a harvest of

pharmaceutical maize. The recombinant maize was accidentally taken to a food

manufacturing facility. However, unlike Scenario 1, the recombinant maize was diluted

with non-recombinant maize, and the protein in the maize was not degraded during

processing. This scenario assumed the same dilution factors as Wolt et al. (2006). Protein expression level in maize kernel was assumed to be 100 mg/kg and 1000 mg/kg of dry grain. For this scenario, it is assumed that one hectare of transgenic maize will yield 5 Mg or 5 metric tons (Wang et al. unpublished). The one hectare field was harvested and taken to a dry mill with a daily milling capacity of 2000 Mg (AgMRC 2003) where the maize was processed into dry-milled food products. Therefore, the percentage of the protein adulterating the maize processed into food products was 0.25% (99.75% dilution). All dry-food products derived from maize (e.g., chips, flour, bran, and starch) from this manufacturing facility were assumed to have the potential to contain transgenic maize and could be eaten by any age group (infant to elderly).

Scenario 3. The third, and least conservative, exposure scenario assumed that the edge of a field expressing transgenic, pharmaceutical-maize pollen drifted to a neighboring non-pharmaceutical maize field (kernels have not been formed yet, fertilization happened when the maize pollen drifted from the transgenic maize field), and the protein was then expressed in 8% of the non-transgenic maize (92% dilution). The 8% expression of transgenic maize in non-transgenic maize was derived from a study conducted by Pla et al. (2006). They determined that gene flow frequency was a function of the distance between the donor transgenic maize and non-transgenic maize in fields adjacent to each other.

The assumptions were that the transgenic maize was planted with no distance between itself and the receptor (non-transgenic) field. The normal distance required between receptor (non-transgenic) maize and source maize (transgenic) is 0.8 km to 1.6

km and a 15.24 m fallow zone around the entire source maize field (Christensen et al. 2004, USDA APHIS 2006). However, this risk assessment does not include any of the required precautions for potential pollen flow. No border rows around the source field site were planted to minimize pollen flow.

The neighboring maize that was assumed to express both transgenic and non-transgenic maize was harvested and taken to a food manufacturing facility. The original dilution factor of 99.75% from Scenario 2, and the second dilution factor of 92% from pollen fertilization to the non-transgenic field were used for this scenario. The two dilution factors were multiplied together for a dilution product that was used in the dietary risk model (see below). The "non-transgenic" field was harvested and taken to a food manufacturing facility and made into dry-milled maize products. All food products derived from maize (e.g., chips, flour, bran, and starch) from this manufacturing facility were assumed to have the potential to contain transgenic maize and could be eaten by any age group (infant to elderly).

Demographic Groups. Five subgroups were used for Scenario 1: adult males (71.8 kg), adult females (60 kg), youth 10-12 years old (40.9 kg), children 5-6 years old (21.1 kg), and toddlers 2-3 years old (14.3 kg). Body weights were obtained from USEPA Exposure Factors Handbook (EPA 1989). Six subgroups were selected for Scenarios 2 and 3: total U.S. population, non-nursing infants, children 1-6 years old, females 13+ (pregnant/not nursing), males 20+ years old, and seniors 55+ years old.

Food Consumption Assumptions. Acute dietary exposures were defined as a single-day event. The Food Commodity Intake Database (FCID) was used with the

Continuing Surveys of Food Intake by Individuals (CSFII) to determine the dietary

consumption of individuals through consumption of food products containing maize for

Scenarios 2 and 3. The FCID is a database that was developed by the USDA for use by

the EPA and other organizations when conducting exposure components of dietary risk

assessments (EPA 2005). The CSFII was created by the USDA and it measures the foods

actually eaten by individuals (USDA 2002). The survey collects data that include

demographics such as household size, income, race, age, sex, and where the food was

purchased, how it was prepared, and where it was eaten. The Dietary Exposure

Evaluation Model (DEEM) uses the information from the CSFII; therefore, DEEM was

used to estimate the inadvertent dietary intake and risk of the therapeutic proteins in food

(Kidwell et al. 2000).

Scenario 1 did not use DEEM because only one food item (tortilla chips) was

included in the exposure estimate, and DEEM cannot calculate dietary exposures based

on one single, branded food item. Scenario 1 assumed that each individual consumed an

entire bag of Tostitos® tortilla chips in one day, regardless of body weight. For

Scenarios 2 and 3, data on body weights and consumption patterns for these

demographic groups were part of the algorithms within the DEEM software. DEEM

calculated dietary exposures to each protein by multiplying the protein expression level

(100 or 1000 ppm) in maize kernels by the dilution factors and by records of

consumption of food products containing maize. Then, DEEM determined percentiles of

dietary exposure to the protein within each demographic subgroup.

Risk Characterization

Risk quotients (RQ's) were calculated for all three scenarios to integrate exposure and effect (toxicity). The dietary exposure values were determined probabilistically within DEEM. The exposure values were then divided by the toxic endpoint for each protein to determine the RQ. Therefore, the RQ, as used here, was the ratio between dietary exposure and the toxic endpoint.

Results and Discussion

Scenario 1

In this scenario with deterministic protein residue and body weight estimates, it was assumed that each individual consumed an entire bag of Tostitos® tortilla chips in one day, regardless of body weight. The lowest RQ with aprotinin expression at 100 ppm was for adult males (0.13) and the highest was for toddlers (0.67) (Table 1). For aprotinin expression at 1000 ppm, the RQ values would increase by a factor of 10. Therefore, all the RQ values for 1000 ppm expression of aprotinin in maize exceeded 1, meaning that dietary exposure was greater than the toxic endpoint.

Gastric lipase RQ's for scenario 1 did not exceed an RQ of 1 for either 100 ppm expression or 1000 ppm expression. The RQ values ranged from 0.001 to 0.003, for 100 ppm expression, and ranged from 0.01 to 0.03, for 1000 ppm expression (Table 1).

LT-B, like aprotinin, had RQ's greater than 1. The RQ values ranged from 32.44 to 167.25, for 100 ppm expression, and ranged from 324.37 to 1672.5 for 1000 ppm expression (Table 1).

Scenario 2

Scenario 2 did not result in RQ's greater than 1 for any of the three proteins. The range of RQ's for aprotinin expression at 100 ppm at the 50th percentile of dietary exposure was 1×10^{-6} to 1×10^{-5}, at the 95th percentile the range was 2×10^{-4} to 3×10^{-5}, and at the 99th percentile the range was 6×10^{-5} to 2×10^{-4} (Table 2).

The range of RQ's for gastric lipase expression at 100 ppm at the 50th percentile was 0 to 5×10^{-8}, at the 95th percentile the range was 1.4×10^{-7} to 6.7×10^{-7}, and at the 99th percentile the range was 2×10^{-7} to 1×10^{-6} (Table 3).

The RQ range for LT-B expression at 100 ppm at the 50th percentile was 0 to 3×10^{-4}, at the 95th percentile the range was 0.009 to 0.042, and at the 99th percentile the range was 0.015 to 0.08 (Table 4).

The range of RQ's was 10-fold higher for expression levels of 1000 ppm for all proteins. The RQ's for all three proteins did not exceed a value of one. The dilution factor reduced the dietary exposure of the therapeutic proteins, and this can be seen in the RQ values in Tables 2-4.

Scenario 3

Scenario 3 did not result in RQ's greater than 1 for any of the three proteins. The RQ range for aprotinin expression at 100 ppm at the 50th percentile was 0 to 3×10^{-7}, at

the 95th percentile the range was 3×10^{-6} to 1×10^{-5}, and at the 99th percentile the range was 5×10^{-6} to 3×10^{-5} (Table 5).

The RQ range for gastric lipase expression at 100 ppm at the 50th percentile was 0 to 1×10^{-9}, at the 95th percentile the range was 1×10^{-8} to 5×10^{-8}, and at the 99th percentile the range was 2×10^{-8} to 5×10^{-5} (Table 6).

The range of RQ's for LT-B expression at 100 ppm at the 50th percentile was 0 to $6 \times 10-5$, at the 95th percentile the range was 0.0007 to 0.003, and at the 99th percentile the range was 0.001 to 0.006 (Table 7).

The human-health risk assessments revealed that the most conservative scenario produced higher RQ's than the other two scenarios. The dietary risks from Scenario 1 were at least 1000 times greater than Scenario 2 and at least 14,000 times greater than Scenario 3. Scenario 1 did not have a dilution factor applied to it; therefore, the individuals were consuming the highest amount of protein possible when the protein was expressed in maize and made directly into the single food product, tortilla chips.

The RQ's decreased by at least three orders of magnitude in Scenarios 2 and 3 compared to Scenario 1 because of the dilution of the transgenic maize with non-transgenic maize. Scenario 2's dilution factor came from the mixing of the non-transgenic maize with the transgenic maize. Scenario 3's dilution factor came from transgenic-maize pollen fertilizing the non-transgenic maize and then harvesting the non-transgenic maize (partially transgenic) and mixing it with other non-transgenic maize. Although still using highly conservative assumptions, each of these more refined dietary

exposure estimates did not exceed toxic endpoints, even at the 99th percentile of exposure.

The greatest difference in risk was attributable to the differences in toxic endpoints of the proteins. Although the protein expression and dietary consumption was assumed to be the same for all proteins, dietary risks between the proteins varied by more than 56,000 times, reflecting the toxic endpoint differences between gastric lipase (1000 mg/kg BW) and LT-B (0.016 mg/kg BW).

An adult male would have to consume about 2.8 kg per day of aprotinin maize expressed at 100 ppm (Scenario 1) to reach the toxic endpoint value, which is the same as an RQ value of 1. For gastric lipase, this value would be 700 kg of maize per day, and for LT-B this value would be 11.2 g of maize per day. For Scenario 2, an adult male would have to consume 1,120 kg of aprotinin, 280,000 kg of gastric lipase, and 4.48 kg of LT-B maize per day to reach each protein's respective toxic endpoint. Because of further dilution due to pollen flow in Scenario 3, an adult male would have to consume 14,000 kg of aprotinin, 3,500,000 kg of gastric lipase, and 56 kg of LT-B per day to reach each protein's respective toxic endpoint. Daily maize consumption per capita is 86 g per day in the US (EPA 2003; Wolt et al. 2006).

Wolt et al. (2006) assessed the risk of unintended antigen occurrence in food by establishing scenarios using maize-expressed LT-B as a case study. The scenarios in this risk assessment are similar to the scenarios in Wolt et al. (2006) in that they assumed a high end consumer group (13 to 19 year old males) consuming three servings of tortilla chips made solely from maize expressing LT-B. This is a worst case scenario similar to

Scenario 1 of this risk assessment with the only differences being more age groups were considered and the entire bag of tortilla chips was consumed. Wolt et al. (2006) considered dilution scenarios in which the LT-B maize was mixed with non-transgenic maize and made into tortilla chips and then consumed by high-end consumers. Scenario 2 of this risk assessment used their dilution factors, but then assumed that the diluted maize was processed into dry milled corn products (e.g., chips, flour, bran, and starch) as well.

Howard and Donnelly (2004) proposed a quantitative safety assessment model for aprotinin produced in transgenic maize. Similar to this risk assessment, they assumed an expression level of 100 mg/kg and no inactivation or loss of function of the protein in transgenic maize. However, their toxic endpoint value of 125 mg/kg BW (dog NOEL after injection) was 31 times less toxic than our value of 4 mg/kg BW (loading dose of aprotinin when administered to human patients undergoing cardio-pulmonary bypass surgery). The endpoint of 4 mg/kg BW is more appropriate for this risk assessment because the loading dose is a value that has been determined based on human exposure to aprotinin. This dosage has resulted in deleterious effects in patients and is a more conservative value than the dog NOEL.

Howard and Donnelly (2004) assumed a 70 kg person consumed a 16-oz box of cereal with 90% of protein activity lost due to processing (heat inactivation) with protein expression at 100 ppm. Their risk assessment is much different from this risk assessment because they use a different approach to determine the level of risk. They did not assume multiple scenarios, instead assumed a case in which the protein is

degraded during processing. This is different from this risk assessment where dilution is considered by inadvertent mixing with non-transgenic maize. They did not use DEEM to determine the amount consumed daily by individuals, but instead determined the amount consumed by using and ingestion rate value based on an inactivation factor (empirically based on the normal preparation of corn grain for food uses).

Both this risk assessment and that of Howard and Donnelly (2004) assumed that aprotinin was as active orally by injection. Howard and Donnelly (2004) concluded that no confinement is required to achieve safety with respect to toxicity of aprotinin, but with regard to aprotinin's potential as an allergen, they recommend that confinement practices be used.

From the findings in the risk assessment presented in this thesis, confinement would be necessary based on uncertainties regarding repeated dietary exposures and allergenicity. Also, when aprotinin is expressed at 1000 mg/kg, the risk quotients for all age groups would be above levels of concern for Scenario 1 (Table 1).

The scenarios applied to this risk assessment did not incorporate USDA permit guidelines (USDA APHIS 2006). Had those guidelines been taken into account with this risk assessment (e.g., distance between fields, planting time, and fallow zone), exposure and subsequent dietary risks for all scenarios would be much smaller.

The uncertainties associated with this risk assessment included the expression level of the protein in maize and the endpoint values for aprotinin and LT-B. Gastric lipase has an experimentally derived NOEL that can be used for risk assessment. The endpoint value used for aprotinin came from the loading dose that is administered to

patients undergoing cardio pulmonary bypass surgery. This dosage has resulted in deleterious effects in patients, and therefore does not constitute an acceptable NOEL for risk assessment. The endpoint value for LT-B is from a study in humans and represents a dosage in which no effects were observed, but that study did not experimentally derive a NOEL.

Because differences in toxic endpoints were responsible for the greatest differences in dietary risk, more precise knowledge is needed regarding toxicity of these pharmaceutical proteins as it relates to oral ingestion via adventitious presence in food. Typically, NOEL's are not established for pharmaceuticals. However, because of the possibility of production of pharmaceutical proteins in crop species and unintended occurrence in food, NOEL's should be established. Further, the NOEL's should be determined for the appropriate route of exposure—oral ingestion. For aprotinin, there is no publicly available toxicity information for the oral route of exposure. Determination of an acute, oral NOEL would substantially reduce uncertainty associated with dietary risk of that protein.

Another uncertainty factor is allergenicity. The interaction of LT-B with the immune system is not well understood, and it is unknown if LT-B could cause indirect toxicity based on its adjuvant properties. As an adjuvant, it potentially could heighten response to allergens in the diet (Wolt et al. 2006). Allergic reactions have been observed after re-exposure to high-dose aprotinin. Aprotinin is immunogenic and induces immunoglobulin G and E (IgG and IgE) responses, but usually only after a secondary exposure (Beierlein et al. 2000; Milano et al. 2002). It is possible for

aprotinin to cause adverse effects after repeated applications based on its antigenic properties, and a prick test is recommended prior to administration of aprotinin. This risk assessment demonstrates that human-health risks vary dramatically and depend on factors such as the specific pharmaceutical protein, protein expression, and exposure scenario. Previously, Nap et al. (2003), Peterson and Arntzen (2004), Kirk et al. (2005), and Ma et al. (2005) argued for the need to assess risks from plant-based pharmaceuticals on a case-by-case basis. The assessment presented here, using three different pharmaceutical proteins, reinforces those arguments and demonstrates the need for case-by-case assessments.

Table 1. Human dietary risk assessment results from exposure scenario 1.

Subgroup (age in years)	Aprotinin RQ[a] (100 ppm[b])	Aprotinin RQ (1000 ppm)	Gastric Lipase RQ (100 ppm)	Gastric Lipase RQ (1000 ppm)	LT-B RQ (100 ppm)	LT-B RQ (1000 ppm)
Adults - males; 73.8 kg	0.13	1.3	0.001	0.01	32.44	324.38
Adults - females; 60.6 kg	0.16	1.58	0.001	0.01	39.5	395
Youth (10-12); 40.9 kg	0.23	2.34	0.001	0.01	58.5	585
Children (5-6); 21.15 kg	0.45	4.52	0.002	0.02	113.06	1130.63
Toddlers (2-3); 14.3 kg	0.67	6.69	0.003	0.03	167.25	1672.5

[a] RQ = Risk Quotient [dietary exposure ÷ toxic endpoint]
[b] ppm = mg/kg dry kernel weight

Table 2. Aprotinin human dietary risk assessment results from exposure scenario 2.

	50th percentile		95th percentile		99th percentile	
	Exposure (mg/kg BW)	RQ [a]	Exposure (mg/kg BW)	RQ	Exposure (mg/kg BW)	RQ
US Population	0.000007	1.8×10^{-6}	0.000277	6.9×10^{-5}	0.000618	2×10^{-4}
Infants [b]	0	0	0.00067	0.00017	0.001252	3×10^{-4}
Children [c]	0.000054	1.4×10^{-5}	0.000614	0.00015	0.001108	3×10^{-4}
Females 13+ [d]	0.000007	1.8×10^{-6}	0.000227	5.7×10^{-5}	0.000297	7×10^{-5}
Males 20+	0.000004	1×10^{-6}	0.000182	4.6×10^{-5}	0.000445	1×10^{-4}
Seniors 55+	0.000004	1×10^{-6}	0.000138	3.5×10^{-5}	0.000247	6×10^{-5}

[a] RQ = Risk Quotient [dietary exposure ÷ toxic endpoint]
[b] non-nursing (<1 yr. old)
[c] 1-6 yr. old
[d] pregnant not nursing
Expression level = 100 ppm

	50th percentile		95th percentile		99th percentile	
	Exposure [mg/kg BW]	RQ [a]	Exposure [mg/kg BW]	RQ	Exposure [mg/kg BW]	RQ
US Population	0.000067	1.7×10^{-5}	0.002772	0.00069	0.006183	0.002
Infants [b]	0	0	0.006696	0.00167	0.012523	0.003
Children [c]	0.000539	0.00013	0.006141	0.00154	0.011083	0.003
Females 13+ [d]	0.000072	1.8×10^{-5}	0.002265	0.00057	0.002974	7×10^{-4}
Males 20+	0.000042	1.1×10^{-5}	0.001816	0.00045	0.004448	0.001
Seniors 55+	0.000039	9.8×10^{-6}	0.001384	0.00035	0.00247	6×10^{-4}

[a] RQ = Risk Quotient [dietary exposure ÷ toxic endpoint]
[b] non-nursing (<1 yr. old)
[c] 1-6 yr. old
[d] pregnant not nursing
Expression level = 1000 ppm

Table 3. Gastric lipase human dietary risk assessment results from exposure scenario 2.

	50th percentile		95th percentile		99th percentile	
	Exposure [mg/kg BW]	RQ [a]	Exposure [mg/kg BW]	RQ	Exposure [mg/kg BW]	RQ
US Population	0.000007	7×10^{-9}	0.000277	3×10^{-7}	0.000618	6.18×10^{-7}
Infants [b]	0	0	0.00067	7×10^{-7}	0.001252	1.25×10^{-6}
Children [c]	0.000054	5×10^{-8}	0.000614	6×10^{-7}	0.001108	1.11×10^{-6}
Females 13+ [d]	0.000007	7×10^{-9}	0.000227	2×10^{-7}	0.000297	2.97×10^{-7}
Males 20+	0.000004	4×10^{-9}	0.000182	2×10^{-7}	0.000445	4.45×10^{-7}
Seniors 55+	0.000004	4×10^{-9}	0.000138	1×10^{-7}	0.000247	2.47×10^{-7}

[a] RQ = Risk Quotient [dietary exposure ÷ toxic endpoint]
[b] non-nursing (<1 yr. old)
[c] 1-6 yr. old
[d] pregnant not nursing
Expression level = 100 ppm

	50th percentile		95th percentile		99th percentile	
	Exposure [mg/kg BW]	RQ [a]	Exposure [mg/kg BW]	RQ	Exposure [mg/kg BW]	RQ
US Population	0.000067	7×10^{-8}	0.002772	3×10^{-6}	0.006183	6.18×10^{-6}
Infants [b]	0	0	0.006696	7×10^{-6}	0.012523	1.25×10^{-5}
Children [c]	0.000539	5×10^{-7}	0.006141	6×10^{-6}	0.011083	1.11×10^{-5}
Females 13+ [d]	0.000072	7×10^{-8}	0.002265	2×10^{-6}	0.002974	2.97×10^{-6}
Males 20+	0.000042	4×10^{-8}	0.001816	2×10^{-6}	0.004448	4.45×10^{-6}
Seniors 55+	0.000039	4×10^{-8}	0.001384	1×10^{-6}	0.00247	2.47×10^{-6}

[a] RQ = Risk Quotient [dietary exposure ÷ toxic endpoint]
[b] non-nursing (<1 yr. old)
[c] 1-6 yr. old
[d] pregnant not nursing
Expression level = 1000 ppm

Table 4. LT-B human dietary risk assessment results from exposure scenario 2.

	50th percentile		95th percentile		99th percentile	
	Exposure [mg/kg BW]	RQ [a]	Exposure [mg/kg BW]	RQ	Exposure [mg/kg BW]	RQ
US Population	0.000007	0.0004	0.000277	0.017	0.000618	0.0386
Infants [b]	0	0	0.00067	0.042	0.001252	0.0783
Children [c]	0.000054	0.0034	0.000614	0.038	0.001108	0.0693
Females 13+ [d]	0.000007	0.0004	0.000227	0.014	0.000297	0.0186
Males 20+	0.000004	0.0003	0.000182	0.011	0.000445	0.0278
Seniors 55+	0.000004	0.0003	0.000138	0.009	0.000247	0.0154

[a] RQ = Risk Quotient [dietary exposure ÷ toxic endpoint]
[b] non-nursing (<1 yr. old)
[c] 1-6 yr. old
[d] pregnant not nursing
Expression level = 100 ppm

	50th percentile		95th percentile		99th percentile	
	Exposure [mg/kg BW]	RQ [a]	Exposure [mg/kg BW]	RQ	Exposure [mg/kg BW]	RQ
US Population	0.000067	0.0042	0.002772	0.173	0.006183	0.3864
Infants [b]	0	0	0.006696	0.419	0.012523	0.7827
Children [c]	0.000539	0.0337	0.006141	0.384	0.011083	0.6927
Females 13+ [d]	0.000072	0.0045	0.002265	0.142	0.002974	0.1859
Males 20+	0.000042	0.0026	0.001816	0.114	0.004448	0.278
Seniors 55+	0.000039	0.0024	0.001384	0.087	0.00247	0.1544

[a] RQ = Risk Quotient [dietary exposure ÷ toxic endpoint]
[b] non-nursing (<1 yr. old)
[c] 1-6 yr. old
[d] pregnant not nursing
Expression level = 1000 ppm

Table 5. Aprotinin human dietary risk assessment results from exposure
scenario 3.

	50th percentile		95th percentile		99th percentile	
	Exposure [mg/kg BW]	RQ [a]	Exposure [mg/kg BW]	RQ	Exposure [mg/kg BW]	RQ
US Population	0.000001	3×10^{-7}	0.000022	6×10^{-6}	0.000049	1×10^{-5}
Infants [b]	0	0	0.000054	1×10^{-5}	0.0001	3×10^{-5}
Children [c]	0.000004	1×10^{-6}	0.000049	1×10^{-5}	0.000089	2×10^{-5}
Females 13+ [d]	0.000001	3×10^{-7}	0.000018	5×10^{-6}	0.000024	6×10^{-6}
Males 20+	0	0	0.000015	4×10^{-6}	0.000036	9×10^{-6}
Seniors 55+	0	0	0.000011	3×10^{-6}	0.00002	5×10^{-6}

[a] RQ = Risk Quotient [dietary exposure ÷ toxic endpoint]
[b] non-nursing (<1 yr. old)
[c] 1-6 yr. old
[d] pregnant not nursing
Expression level = 100 ppm

	50th percentile		95th percentile		99th percentile	
	Exposure [mg/kg BW]	RQ [a]	Exposure [mg/kg BW]	RQ	Exposure [mg/kg BW]	RQ
US Population	0.000005	1×10^{-6}	0.000222	6×10^{-5}	0.000495	0.0001
Infants [b]	0	0	0.000536	0.0001	0.001002	0.0003
Children [c]	0.000043	1×10^{-5}	0.000491	0.0001	0.000887	0.0002
Females 13+ [d]	0.000006	2×10^{-6}	0.000181	5×10^{-5}	0.000238	6×10^{-5}
Males 20+	0.000003	8×10^{-7}	0.000145	4×10^{-5}	0.000356	9×10^{-5}
Seniors 55+	0.000003	8×10^{-7}	0.000111	3×10^{-5}	0.000198	5×10^{-5}

[a] RQ = Risk Quotient [dietary exposure ÷ toxic endpoint]
[b] non-nursing (<1 yr. old)
[c] 1-6 yr. old
[d] pregnant not nursing
Expression level = 1000 ppm

Table 6. Gastric lipase human dietary risk assessment results from exposure scenario 3.

	50th percentile		95th percentile		99th percentile	
	Exposure [mg/kg BW]	RQ[a]	Exposure [mg/kg BW]	RQ	Exposure [mg/kg BW]	RQ
US Population	0.000001	1×10^{-9}	0.000022	2×10^{-8}	0.000049	5×10^{-5}
Infants[b]	0	0	0.000054	5×10^{-8}	0.0001	1×10^{-7}
Children[c]	0.000004	4×10^{-9}	0.000049	5×10^{-8}	0.000089	9×10^{-8}
Females 13+[d]	0.000001	1×10^{-9}	0.000018	2×10^{-8}	0.000024	2×10^{-8}
Males 20+	0	0	0.000015	2×10^{-8}	0.000036	4×10^{-8}
Seniors 55+	0	0	0.000011	1×10^{-8}	0.00002	2×10^{-8}

[a] RQ = Risk Quotient [dietary exposure ÷ toxic endpoint]
[b] non-nursing (<1 yr. old)
[c] 1-6 yr. old
[d] pregnant not nursing
Expression level = 100 ppm

	50th percentile		95th percentile		99th percentile	
	Exposure [mg/kg BW]	RQ[a]	Exposure [mg/kg BW]	RQ	Exposure [mg/kg BW]	RQ
US Population	0.000005	5×10^{-9}	0.000222	2×10^{-7}	0.000495	5×10^{-7}
Infants[b]	0	0	0.000536	5×10^{-7}	0.001002	1×10^{-6}
Children[c]	0.000043	4×10^{-8}	0.000491	5×10^{-7}	0.000887	9×10^{-7}
Females 13+[d]	0.000006	6×10^{-9}	0.000181	2×10^{-7}	0.000238	2×10^{-7}
Males 20+	0.000003	3×10^{-9}	0.000145	1×10^{-7}	0.000356	4×10^{-7}
Seniors 55+	0.000003	3×10^{-9}	0.000111	1×10^{-7}	0.000198	2×10^{-7}

[a] RQ = Risk Quotient [dietary exposure ÷ toxic endpoint]
[b] non-nursing (<1 yr. old)
[c] 1-6 yr. old
[d] pregnant not nursing
Expression level = 1000 ppm

Table 7. LT-B human dietary risk assessment results from exposure scenario 3.

	50th percentile		95th percentile		99th percentile	
	Exposure [mg/kg BW]	RQ [a]	Exposure [mg/kg BW]	RQ	Exposure [mg/kg BW]	RQ
US Population	0.000001	6×10^{-5}	0.000022	0.0014	0.000049	0.00306
Infants [b]	0	0	0.000054	0.0034	0.0001	0.00625
Children [c]	0.000004	0.0003	0.000049	0.0031	0.000089	0.00556
Females 13+ [d]	0.000001	6×10^{-5}	0.000018	0.0011	0.000024	0.0015
Males 20+	0	0	0.000015	0.0009	0.000036	0.00225
Seniors 55+	0	0	0.000011	0.0007	0.00002	0.00125

[a] RQ = Risk Quotient [dietary exposure ÷ toxic endpoint]
[b] non-nursing (<1 yr. old)
[c] 1-6 yr. old
[d] pregnant not nursing
Expression level = 100 ppm

	50th percentile		95th percentile		99th percentile	
	Exposure [mg/kg BW]	RQ [a]	Exposure [mg/kg BW]	RQ	Exposure [mg/kg BW]	RQ
US Population	0.000005	0.0003	0.000222	0.0139	0.000495	0.03094
Infants [b]	0	0	0.000536	0.0335	0.001002	0.06263
Children [c]	0.000043	0.0027	0.000491	0.0307	0.000887	0.05544
Females 13+ [d]	0.000006	0.0004	0.000181	0.0113	0.000238	0.01488
Males 20+	0.000003	0.0002	0.000145	0.0091	0.000356	0.02225
Seniors 55+	0.000003	0.0002	0.000111	0.0069	0.000198	0.01238

[a] RQ = Risk Quotient [dietary exposure ÷ toxic endpoint]
[b] non-nursing (<1 yr. old)
[c] 1-6 yr. old
[d] pregnant not nursing
Expression level = 1000 ppm

CHAPTER 3

AN ECOLOGICAL RISK ASSESSMENT

FOR PLANT-BASED PHARMACEUTICALS

Abstract

Pharmaceuticals using genetic engineering have been made through protein expression in bacterial, fungal, and mammalian cell cultures. Plant-based pharmaceuticals offer a cleaner method of producing a protein for drug manufacturing than traditional methods because plants are free of mammalian infectious agents. Plant-based pharmaceuticals in the open environment have the potential of intra-and inter-species gene flow, protein exposure to the public and non-target organisms, and they also have the potential to contaminate livestock feed. Ecological risk assessments evaluate the likelihood that adverse ecological effects may occur or are occurring as a result of exposure to one or more stressors. This study used probabilistic approaches to quantify the ecological risks associated with three pharmaceutical proteins produced in field-grown maize. The ecological risk assessment for plant-based pharmaceuticals was conducted for four receptor species used as surrogates for a wider range of species. Body weights and maize consumption rates for each species were modeled from currently available information and used to calculate the exposure based on different expression levels of the proteins. The two expression levels were 100 mg/kg dry kernel weight

(ppm) and 1000 ppm for all three proteins (aprotinin, gastric lipase, and LT-B). The acute dietary exposure for the receptor species was a single-day event, where the total maize consumption came from the recombinant maize. The ecological risk assessment demonstrated that risks will vary between species and between proteins, based primarily on differences in toxic endpoint and consumption rates. It also shows the utility of probabilistic, quantitative risk assessment methodologies and supports the human dietary risk assessment by demonstrating the importance of assessing risks from plant-based pharmaceuticals on a case-by-case basis.

Introduction

Pharmaceuticals using genetic engineering have been made through protein expression in bacterial, fungal, and mammalian cell cultures. Genetic engineering has made it possible to use plants as factories for pharmaceutical protein production. Plant-based pharmaceuticals are made by inserting a segment of DNA that encodes the protein of choice into the plant cells. The plants or the plant cells are essentially factories used to produce the desired proteins and are only grown for the purpose of pharmaceutical applications (Shama and Peterson 2004).

Plants expressing pharmaceutical proteins are currently being grown in field environments (although on few hectares) throughout the United States and other countries. Plant-based pharmaceuticals have the benefits of being less expensive to produce and potentially being more readily available to individuals in remote locations. It is also a cleaner method of producing a protein for drug manufacturing because plants are free of mammalian and avian infectious agents.

Maize is an attractive vehicle for cloned vaccine antigens and other pharmaceutical proteins because it is capable of being processed into several palatable forms. Maize-based antigens are also inexpensive to produce and scale up. The distribution of the cloned antigen within the maize kernels is homogeneous, allowing for a reproducible dose (Fischer and Emans 2000). Such a vaccine would be well suited for developing nations where refrigeration during storage and distribution is often difficult, and syringes and other supplies for immunization are expensive and unsafe. The proof-of-concept for transgenic plant vaccines has been demonstrated in farm animal models and extensive trials are underway with promising results (Ma et al. 2003; Peterson and Arntzen 2004).

However, there are concerns about growing therapeutic proteins in the open environment. Plant-based pharmaceuticals in the environment have the potential for intra-and inter-species gene flow, protein exposure to the public and non-target organisms, contamination of livestock feed, and water contamination (Peterson and Arntzen 2004; Freese 2005).

Plant-based pharmaceutical production requires regulatory involvement of both the U.S. Department of Agriculture (USDA) and the U.S. Food and Drug Agency (USFDA), and this often involves risk assessment approaches. A risk assessment ensures a robust, transparent, and science-based process in which the assumptions and uncertainties associated with the assessment are considered and presented. Ecological risk assessments evaluate the likelihood that adverse ecological effects may occur or are occurring as a result of exposure to one or more stressors (EPA 1992a). Much attention

has been directed toward the use of probabilistic risk assessment techniques that statistically quantify ecological risks as well as the associated uncertainty and variability in the subsequent risk conclusions (SETAC 1994; EPA 1999). Risk assessment paradigms for genetically engineered plants do not differ in principle from those for other technological risks. Therefore, probabilistic approaches to quantify risks for crop biotechnology should be used for conducting ecological risk assessments, where appropriate (NRC 2000; Wolt and Peterson 2000; Wolt et al. 2003).

The objective of this study was to use probabilistic approaches to quantify the ecological risks associated with three pharmaceutical proteins produced in field-grown maize. Ecological dietary risks from three proteins occurring in field-grown maize were evaluated based on a single exposure scenario, and the potential risks were compared between species and proteins.

Materials and Methods

Problem Formulation

The ecological risk assessment for plant-based pharmaceuticals was conducted for four receptor species used as surrogates for a wider range of species. The four receptor species chosen were vole (*Microtus pennsylvanicus*), bobwhite quail (*Colinus virginianus*), whitetail deer (*Odocoileus virginianus*), and feeder and slaughter cattle (*Bos taurus)*. Body weights and maize consumption rates for each species were modeled from currently available information and used to calculate the exposure based on different expression levels of the proteins. The two expression levels were 100 mg/kg dry kernel

weight (ppm) and 1000 ppm for all three proteins (aprotinin, gastric lipase, and LT-B). The acute dietary exposure for the receptor species was a single-day event, where the total maize consumption came from the recombinant maize. Body weights, consumption rates, protein expression levels, and total protein exposures were determined using probabilistic, quantitative methods.

Effects Assessment

A complete review of effects (toxicities) from the three proteins is presented in Chapter 1.

Aprotinin

Toxic endpoint. Acute LD_{50} values for mice, rats, dogs, and rabbits have been determined (Bayer 2000). Each study group received IV injections of aprotinin. LD_{50} values were 910 mg/kg body weight (BW) for mice, 700 mg/kg BW for rats, 190 mg/kg BW for dogs, and 70 mg/kg BW for rabbits. The toxicity endpoint for the ecological risk assessment was based on the no-observed-effect-level (NOEL) for dogs injected with aprotinin at 140 mg/kg BW (Trautschold et al. 1967).

Gastric Lipase

Toxicity of Lipase from Genetically Modified *Aspergillus oryzae*

Aquatic Organism Toxicity. Five waterfleas (*Daphnia magna*) were put in a test tube with an aqueous solution of test material at 1 g/L to test the solution's ability to immobilize the *Daphnia* over a 24-hr period under static conditions. No immobilization

was seen after the 24-hr exposure (Greenough et al. 1996). Acute toxicity was also studied in carp (*Cyprinus carpio*). Ten carp were put in an aquarium of an aqueous solution of test material at 1 g/L, with the solution renewed every 48 hours. No mortality was observed after 96 hours (Greenough et al. 1996).

Algal Growth Inhibition Test. An algal growth inhibition test was conducted to observe if there was any inhibitory effect on green algae (*Scenedesmus subspicatus*) when exposed to the test material. The algae were exposed to five concentrations (10-160 mg/L) of test material. The EC_{50} for inhibition of growth after 72 hours was 97 mg/L and the EC_{50} for inhibition of maximum growth rates (24-72 hr) was 99 mg/L. The NOEL was 40 mg/L (Greenough et al. 1996).

Biodegradability. The biodegradability test recorded dissolved organic carbon of 250 mg of test material in sealed flasks. Gastric lipase was tested for its biodegradability in the closed bottle test at concentrations of 5 and 25 mg/L. The material was not readily biodegradable in the closed bottle test, but was not toxic to the microbes present in the medium (Greenough et al. 1996).

Toxic Endpoint. The ingestion NOEL for gastric lipase was 1000 mg/kg BW in rats (Coenen et al. 1997). After a 13-week oral toxicity study in rats by ANZFA (2002), an ingestion NOEL of 830 mg/kg BW was determined. Greenough et al. (1996) also support the 1000 mg/kg BW ingestion NOEL. The maximum NOEL used in their 13-week oral toxicity study in rats was 1350 mg/kg BW. The toxicity endpoint for this risk assessment was 1000 mg/kg BW.

E.coli Heat-Labile Enterotoxin B Subunit (LT-B)

Toxic Endpoint. When LT-B was administered to adult female mice (30 g) through intragastric administration, Guidry et al. (1997) observed a NOEL of 125 μg of LT-B. Therefore, a NOEL of 4.2 mg/kg BW was used for the ecological risk assessment.

Exposure Assessment

Aprotinin Expression in Maize. ProdiGene began commercial scale-up of aprotinin in field-grown maize in 2004. Aprotinin traditionally has been extracted from bovine lungs. ProdiGene's recombinant aprotinin is equivalent to bovine aprotinin (ProdiGene 2002). Zhong et al. (1999) found that aprotinin was at a higher concentration in maize embryonic tissue than in endosperm tissue. Azzoni et al. (2002) reported the expression level of aprotinin produced from transgenic maize seed to be 0.17%, the study by Zhong et al. (1999) reported an expression level of 0.1%.

Gastric Lipase Expression in Maize. Meristem is developing a recombinant mammalian gastric lipase. Recombinant gastric lipase is grown in maize and then purified, extracted, and used in pre-clinical studies. The mammalian lipase from porcine tissue was selected because it is naturally resistant to digestion by stomach acids and maintains a high enzymatic activity after passage through the stomach. The expression level is approximately 1 mg/g kernel, and there is no expression in other plant organs. The expression was observed to be stable over 11 generations (www.meristem-therapeutics.com).

LT-B Expression in Maize. Chikwamba et al. (2002) produced the B subunit of the enterotoxin *E.coli* heat-labile enterotoxin in transgenic maize seed. In their study, a seed-specific promoter regulated the LT-B gene, and no LT-B expression was detected in callus tissues. Data from all LT-B positive kernels in each ear were pooled to determine the mean and standard deviation for each ear. P112 transgenic events showed the highest level of LT-B accumulation in R1 seeds. P112 events carry the construct pRC4-1, in which the endoplasmic reticulum-retention signal sequence SEKDEL was included in the C-terminus of LT-B gene. Inclusion of the SEKDEL motif in the LT-B gene under the regulation of the seed-specific promoter greatly enhanced the LT-B level in P112 events. Compared to transgenic maize plants carrying CaMV 35S promoter/LT-B constructs (P51 and P65), plants carrying zein promoter/LT-B constructs (P77 and P112) were more vigorous in overall performance.

Chikwamba et al. (2003) showed that LT-B protein was detected internally and externally in starch granules of maize. The strong association between the starch granules and the protein gives an effective co-purification of the antigen in the processing of the starch fraction of the corn kernels, thermostability, and resistance to peptic degradation in simulated digestion fluids, which could be a concern if the LT-B maize is inadvertently mixed with food (Chikwamba et al. 2003).

Maize-derived LT-B has been extracted to reveal kernel expression levels of 9.2% of total soluble protein, and 3.7% of total soluble protein has been achieved by using seed specific promoters. As much as 350 μg/g LT-B could be expressed in kernels with the seed specific promoters (Chikwamba et al. 2002).

Protein Expression Assumptions for this Risk Assessment

Expression levels and protein distribution within various parts of the plant is needed to determine potential dietary exposure. Protein expression levels are not known for all of the proteins, but the expression of LT-B is typically 35-50 µg/g of dry seeds (about 4-5 kernels). Improvements in transformation and translation will be made over time and will increase expression levels (Wolt et al. 2006). Therefore, for this ecological risk assessment, 100 mg/kg and 1000 mg/kg are used as the expression levels for all three proteins. Expression of 100 mg/kg represents what is currently being achieved and 1000 mg/kg represents the potential protein expression in the near future (K. Wang, Iowa State Univ., personal communication).

Exposure Assumptions for the Ecological Risk Assessment

Choice of Surrogate Species. The surrogate species were chosen to represent a relatively broad range of species that could potentially ingest the pharmaceutical protein expressed in maize kernels. The surrogate species included: feeder and slaughter cattle, whitetail deer, voles, and bobwhite quail.

Routes, Pathways, and Duration of Exposure. The exposure assumptions for the ecological risk assessment were that the entire daily food intake for each receptor species came from the transgenic maize kernels expressing the therapeutic protein and that this was their sole source of food. The exposure duration was acute, occurring only over one day. Other exposure routes, such as inhalation of pollen and ingestion of leaves, stalks, and roots were not considered because it was assumed that each pharmaceutical protein

would be produced only in the kernels. For cattle and whitetail deer, it was assumed that individuals entered the field and fed on the kernels, as they were mature or maturing while still on the ears. For voles and quail, it was assumed that individuals fed on kernels that may have dropped from the ears during or just before harvest.

Probabilistic Exposure Assessment

A Monte Carlo simulation model (Crystal Ball 2000 ver. 5.2, Decisioneering, Denver, CO) was used to determine protein exposures to surrogate species. The simulation was set to perform 5,000 iterations for distributional analysis using several input assumptions (Table 1). Monte Carlo simulation uses random numbers to measure the effects of uncertainty and variability in a spreadsheet format. The simulation uses a probability distribution function from each input variable to randomly select values and repeatedly selects values based on their frequency of occurrence in the distribution. The variability for each input is taken into account in the output of the model so that the output is itself a distribution and reflects the probability of values that could occur.

The animal body weights and food consumption rates for each surrogate species were defined with a probability distribution based on the most relevant data (see below) (Table 1). To calculate the protein exposure for each surrogate species the following equation was used:

$$IE = (PE * FC) \div BW \qquad\qquad [1]$$

where *IE* = Ingestion Exposure (mg protein/kg body weight/day), *PE* = Protein Expression (mg/kg kernel), *FC* = Food Consumption (kg dry weight of kernels/day), and *BW* = body weight (kg).

Protein Expression

Protein expression was the amount of protein expressed in the maize kernels. Two mean expression levels, 100 mg/kg and 1000 mg/kg, were used for each protein. Expression levels were assumed to be normally distributed with a standard deviation of 20% (Table 1).

Cattle Weight and Feed Consumption Rate

The cattle weights used for both feeder and slaughter cattle were derived from individual weight records separated by state (USDA 2006). The states chosen for this ecological risk assessment were located in the Midwestern US and included Illinois, Nebraska, South Dakota, Missouri, Oklahoma, Kansas, and Iowa. There were no data for slaughter cattle for South Dakota, but all other states were included in the slaughter cattle calculation. A weighted mean and a standard deviation were calculated for both feeder and slaughter cattle.

The weighted mean for body weight was calculated by dividing the number of cattle measured in each state by the total number of cattle measured in all states, and then multiplying this value by the average weight from each state. The final mean was obtained by adding the values. The feeder cattle weight mean was 293.31 ± 3.31 kg, and the slaughter cattle weight mean was 570.43 ± 15.68 kg. The weights for both were normally distributed (Table 1).

The feed consumption rate was determined for both feeder and slaughter cattle by calculating a weighted mean based on values from a corn feeding study (Loerch 1996).

The same equation used to calculate body weight was used to calculate a weighted consumption mean. The feed consumption rate mean was 7.03 ± 0.1 kg for both feeder and slaughter cattle and the data were normally distributed (Table 1).

Vole Weight and Feed Consumption Rate

Vole weight was derived from Krol et al. (2004) by calculating a weighted mean, as described above. The mean weight was 25.6 ± 3.7 g. Food consumption rate was also determined by calculating a weighted mean from the values presented in Krol et al. (2004). The weighted food consumption mean was 6.1 ± 0.8 g. Data for both food consumption rate and body weight were normally distributed (Table 1).

Bobwhite Quail Weight and Feed Consumption Rate

The weight and food consumption rate of the bobwhite quail were obtained from the USEPA Wildlife Exposure Factors Handbook (EPA 1993). The mean body weight was 191.26 ± 4.27 g, and the food consumption rate mean was 14.842 ± 0.0139 g. Data for both food consumption rate and body weight were normally distributed (Table 1).

Whitetail Deer Weight and Feed Consumption Rate

Body weights for whitetail deer were calculated from a range of 40 to 136.36 kg (90 to 300 lbs). The range of distributions for body weight was obtained from Alabama Land Trust (2005) and Sedgwick County Zoo (2006). Weight was modeled as a triangular distribution with 88.64 kg as the likeliest weight (Table 1). Consumption rate was also modeled as a triangular distribution with 2.02 kg as the minimum rate, 2.27 kg

as the likeliest rate, and 2.52 kg as the maximum rate (Table 1). These values were obtained based on the observed 2.27 kg/day food consumption rate (Gallagher and Prince 2003).

Risk Characterization

Risk quotients (RQ's) were calculated for all three scenarios to integrate exposure and effect (toxicity). The dietary exposure values were determined probabilistically by Monte Carlo analysis. The exposure values were then divided by the toxic endpoint for each protein to determine the RQ. Therefore, the RQ, as used here, was the ratio between dietary exposure and the toxic endpoint.

Results and Discussion

Aprotinin risk quotients (RQ's), using the dog NOEL of 140 mg/kg BW for all of the ecological receptors, ranged from 0.009 for slaughter cattle to 0.14 for voles at the 50th percentile of exposure. RQ's ranged from 0.01 for slaughter cattle to 0.17 for voles at the 75th percentile. At the 90th percentile, RQ's ranged from 0.01 for slaughter cattle to 0.2 for voles (Table 2). The vole had a higher RQ than the other surrogates because of the ratio between its body weight and consumption.

Gastric lipase RQ's, using a NOEL of 1000 mg/kg BW, ranged from 0.001 for slaughter cattle to 0.02 for voles at the 50th percentile. At the 75th percentile, the RQ's ranged from 0.001 for slaughter cattle to 0.02 for voles. At the 90th percentile, the RQ's ranged from 0.002 for slaughter cattle to 0.03 for voles (Table 2).

LT-B RQ's, using a mouse NOEL of 4.2 mg/kg BW, ranged from 0.29 for slaughter cattle to 4.81 for voles at the 50th percentile. At the 75th percentile, the RQ's ranged from 0.33 for slaughter cattle to 5.7 for voles. At the 90th percentile, the RQ's ranged from 0.37 for slaughter cattle to 6.53 for voles (Table 2). The RQ's that exceeded a value of 1.0 imply that dietary exposure was greater than the toxic endpoint.

The expression level for this probabilistic risk assessment was 100 ppm. With an expression level of 1000 ppm, the RQ's were 10 times higher. See Table 2 for RQ values. At the 90th percentile with expression of each protein at 1000 ppm, all the species exceeded an RQ of 1.0 for LT-B, and vole exceeded 1.0 at the 90th percentile for aprotinin.

When comparing the RQ's of all the proteins for all the ecological receptors, the RQ's were highest for LT-B. The RQ's under all the percentiles for LT-B were highest for vole and then quail, both exceeding values of 1.0. Gastric lipase had the lowest RQ values for all the ecological receptors. When examining gastric lipase alone, vole, had the highest RQ values for all three percentiles (50th, 75th, 90th). The RQ's for aprotinin were between the values for LT-B and gastric lipase, and, when examining the ecological receptors within aprotinin, vole had the highest RQ values.

The exposure distribution as a result of the Monte Carlo simulation for all of the receptor species, except whitetail deer, showed a normal distribution. The whitetail deer exposure distribution was lognormal,l with a flat kurtosis (4.37) and positive skewness (0.97). This most likely was the result of the triangular distribution and range of body weights used in the analysis. The exposure distribution for whitetail deer revealed that

there would be a low probability of extremely high exposures to proteins expressed in kernels.

A sensitivity analysis using Crystal Ball determines how much a given input assumption affects the result of the forecast. The sensitivity analysis for all the ecological receptors, except whitetail deer, revealed that protein expression level was the input assumption that contributed the most variance to the output distribution. Percent contribution of protein expression level to total variance ranged from 41.7% (whitetail deer) to 98.8% (feeder cattle) (Fig. 3-4). The contribution of consumption to total variance ranged from 0.5% (slaughter cattle) to 28.5% (vole and quail) (Fig. 1, 2, 5), and the contribution of body weight ranged from 0.3% (feeder cattle, quail, and vole) to 55.8% (whitetail deer) (Fig. 1-4). The sensitivity analysis for whitetail deer showed that body weight had the highest sensitivity followed closely by protein expression (Fig. 3).

The uncertainties associated with this risk assessment can be attributed to a few variables. These variables include the toxic endpoint values from each protein, the expression level of each protein in maize, and body weight and consumption values for the ecological receptors. The toxic endpoint values came from a single, deterministic value for a single species for each protein, and these values were extrapolated for the ecological receptors in this risk assessment. At this time, it is unknown whether any of the NOEL's based on dog, mouse, and rat used here can be extrapolated to vole, quail, cattle, or deer. If a 10-fold safety factor was applied to each toxic endpoint, the vole RQ would exceed an RQ of 1.0 for aprotinin. Whitetail deer and cattle would exceed an RQ of 1.0 for LT-B with a 10-fold safety factor applied to the endpoint value.

The greatest contributor to variance in total exposure was protein expression, which is seen clearly from the sensitivity figures (Fig. 1, 2, 4, 5). Protein expression was considered the same for all three proteins, and, currently there is considerable uncertainty with that assumption. Because data on variability in protein expression in kernels generally are not publicly available and field-grown plant-based pharmaceuticals are relatively recent, the protein expression estimates in this model used 20% of the mean as a standard deviation for all proteins. This value was based on a few indications in the literature and expert elicitation (K. Wang, Iowa State Univ., personal communication). Refinements in the risk assessments presented here will be most affected by more accurate estimates of variability in protein expression.

In general, body weight contributed very little to overall variance of total exposure, except for whitetail deer. The body-weight distribution for whitetail deer came from a combination of values, and from these values a triangular distribution was created. To refine the exposure assessment and reduce variance from this input value, more robust measurements of whitetail deer weights would be needed. Daily consumption of maize by each receptor would have refined the exposure assessment as well.

When comparing the RQ values between the proteins, LT-B had values 2.5 orders of magnitude higher when compared to the RQ's for gastric lipase, and 1 to 2 orders of magnitude higher when compared to the RQ's for aprotinin. The RQ values between species for all proteins at the 90th percentile of exposure showed that the vole RQ was 10

times higher. The vole had higher RQ's for all proteins because the body weight-to-food

consumption ratio is small.

Because RQ's for both vole and bobwhite quail exceeded a value of 1.0,

refinements in the risk assessment may be warranted. The toxic endpoint value for birds

is based on the mouse NOEL. A value specifically for birds would greatly improve the

uncertainty of the assessment. The primary area of refinement would be actual

consumption rates of maize kernels per day in the presence of other food choices.

Except for LT-B, this risk assessment revealed that ecological risks (specifically

non-target organism dietary risks) to aprotinin and gastric lipase are unlikely to be

unacceptable. Refinements associated with exposure to LT-B may reduce the RQ below

1.0. The risk assessment demonstrated that risks will vary between species and between

proteins, based primarily on differences in toxic endpoint and consumption rates. It also

showed the utility of probabilistic, quantitative risk assessment methodologies. Finally,

the risk assessment supports the human dietary risk assessment (Chapter 1) by

demonstrating the importance of assessing risks from plant-based pharmaceuticals on a

case-by-case basis.

Table 8. Input distributions for the probabilistic exposure analysis.

Input distribution	Distribution type	Parameter	Value	Unit
Protein expression level	normal	Mean	100	Mg/kg
		SD	20	
		Lower Bound	0	
Feeder cattle				
Consumption	normal	Mean	7.03	kg/day
		SD	0.1	kg/day
		Lower Bound	0	
Weight	normal	Mean	293.31	Kg
		SD	3.31	Kg

Table 8 continued.

		Lower Bound	0	
Slaughter cattle				
Consumption	normal	Mean	7.03	kg/day
		SD	0.1	kg/day
		Lower Bound	0	
Weight	normal	Mean	570.43	Kg
		SD	15.68	Kg
		Lower Bound	0	
Vole				
Consumption	normal	Mean	6.1	g/day
		SD	0.8	g/day
		Lower Bound	0	
Weight	normal	Mean	29.2	G
		SD	0.4	G
		Lower Bound	0	
Whitetail deer				
Consumption	triangular	Minimum	2.02	kg/day
		Likeliest	2.27	kg/day
		Maximum	2.52	kg/day
Weight	triangular	Minimum	40.9	Kg
		Likeliest	88.64	Kg
		Maximum	136.36	Kg
Bobwhite quail				
Consumption	normal	Mean	14.84	kg/day
		SD	0.01	kg/day
		Lower Bound	0	
Weight	normal	Mean	191.26	Kg
		SD	4.27	Kg
		Lower Bound	0	

Table 9. Risk quotients from the probabilistic ecological risk assessment.

	Aprotinin NOEL[a] = 140			Gastric Lipase NOEL = 1000			LT-B NOEL = 4.2		
					Percentiles				
	50th	75th	90th	50th	75th	90th	50th	75th	90th
Quail	0.06[b]	0.06	0.07	0.01	0.01	0.01	1.83	2.09	2.34
Vole	0.14	0.17	0.20	0.02	0.02	0.03	4.81	5.70	6.53
Deer	0.02	0.02	0.03	0.003	0.003	0.004	0.61	0.75	0.92
Feeder Cattle	0.02	0.02	0.02	0.002	0.003	0.003	0.57	0.64	0.72
Slaughter Cattle	0.01	0.01	0.01	0.001	0.001	0.002	0.29	0.33	0.92

[a]NOEL = mg/kg BW
[b]Risk Quotient = Ingestion Exposure ÷ NOEL
Protein expression level = 100 mg/kg

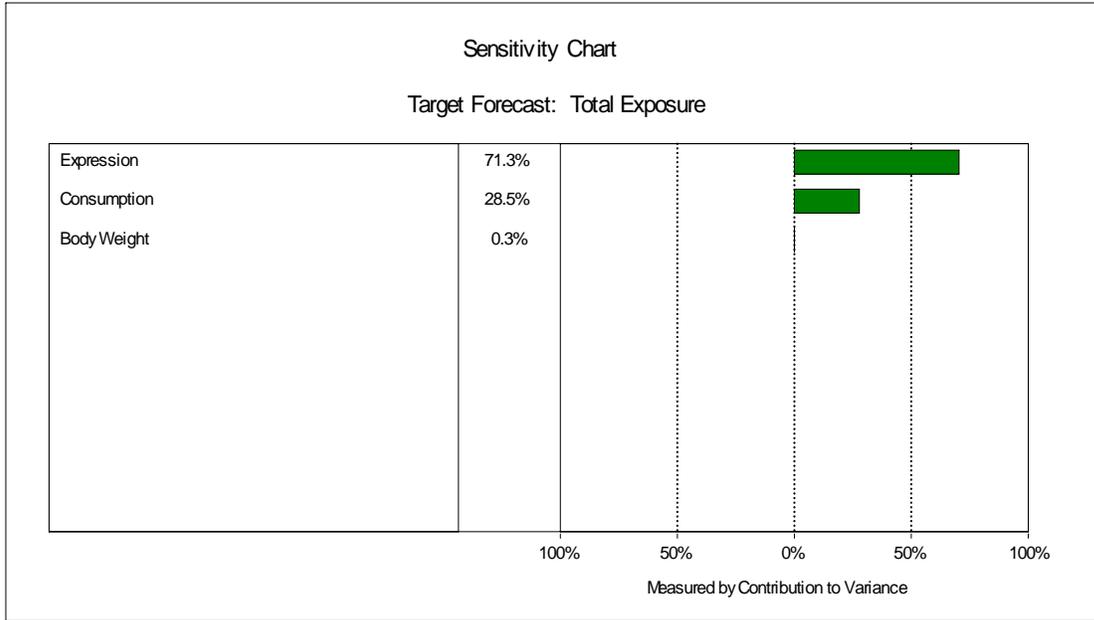Figure 3. Bobwhite quail sensitivity chart from the Monte Carlo exposure simulation.

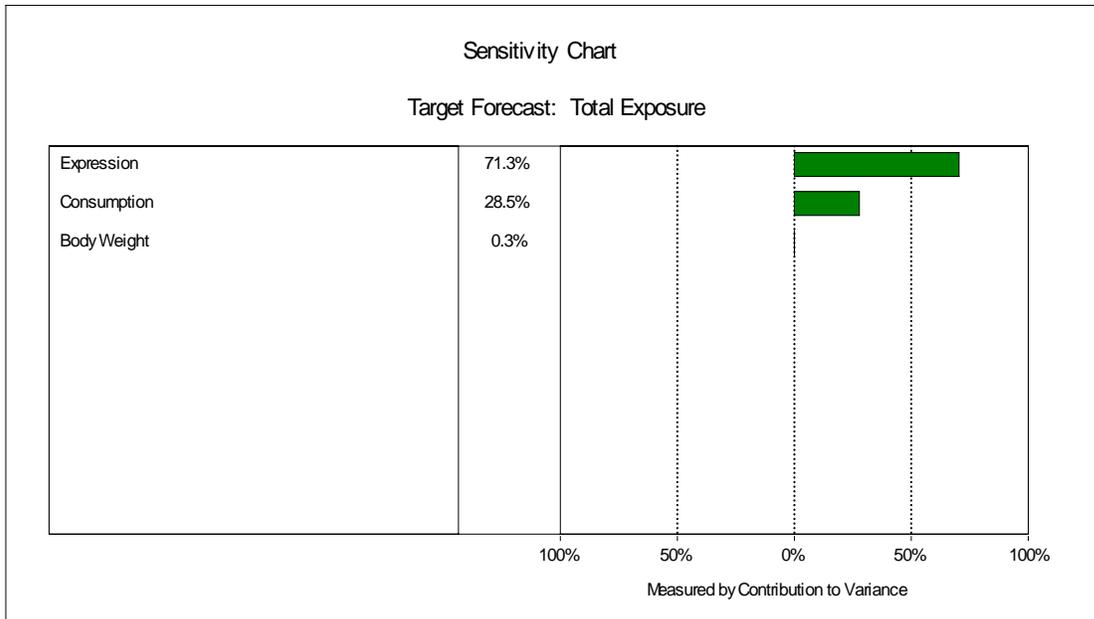Figure 4. Vole sensitivity chart from the Monte Carlo exposure simulation.

Figure 5. Whitetail deer sensitivity chart from the Monte Carlo exposure simulation.
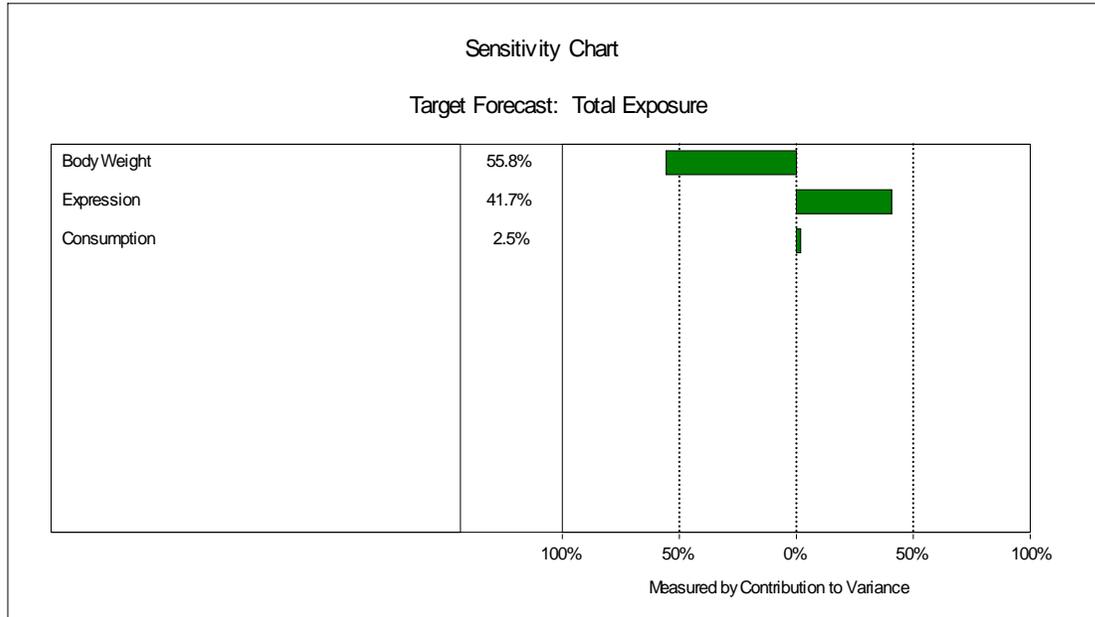

Figure 6. Feeder cattle sensitivity chart from the Monte Carlo exposure simulation.
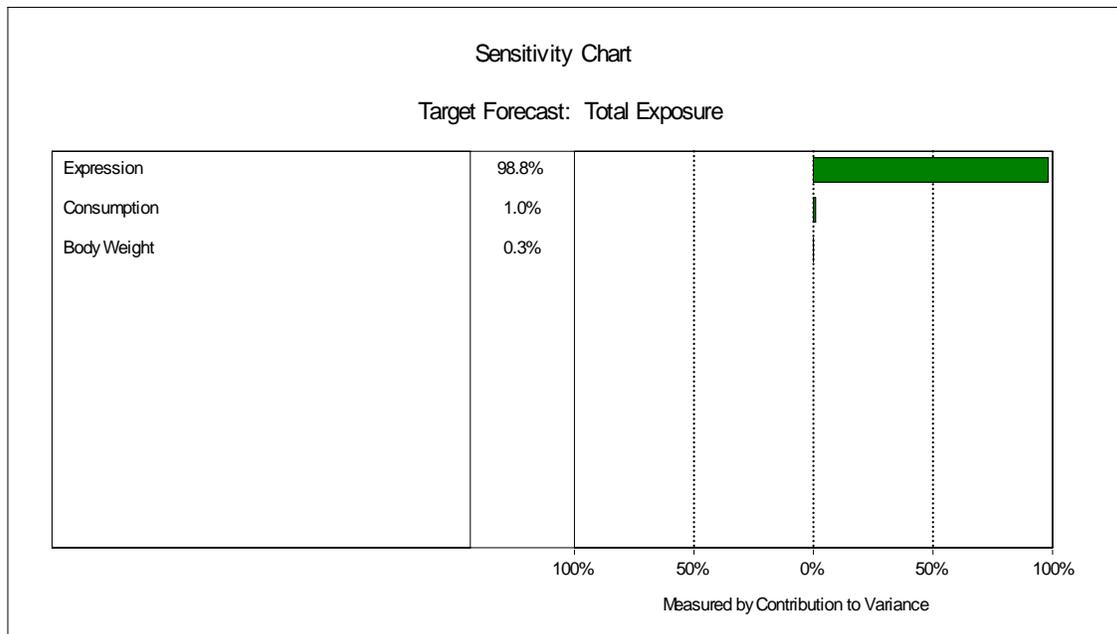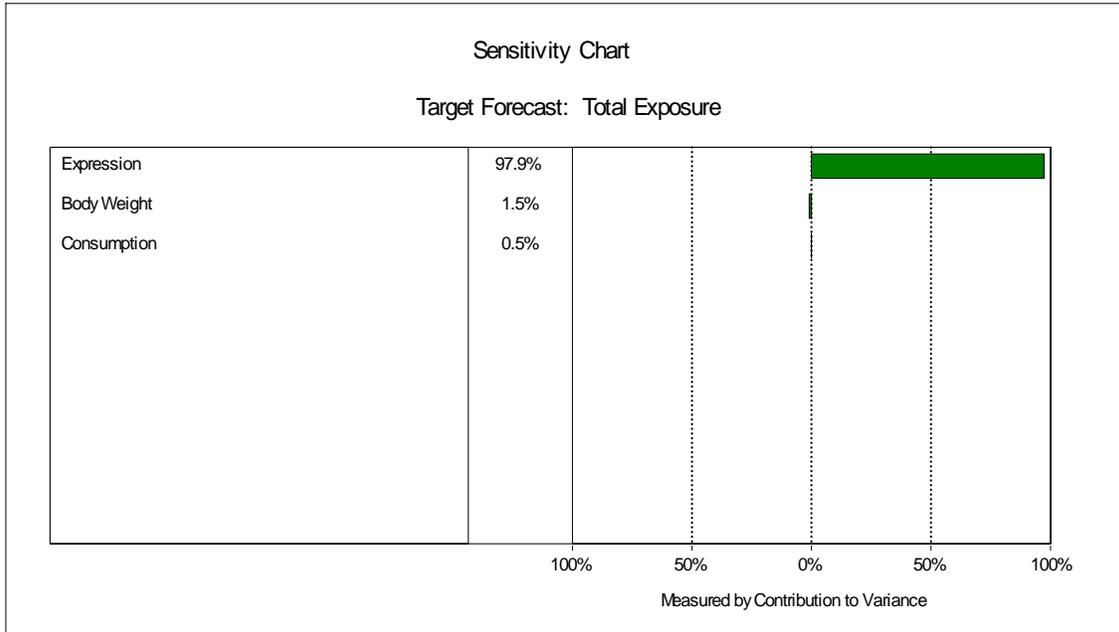
Figure 7. Slaughter cattle sensitivity chart from the Monte Carlo exposure simulation.

CHAPTER 4

A QUALITATIVE COMPARATIVE RISK ASSESSMENT BETWEEN A PLANT-

CELL DERIVED AND CONVENTIONALLY DERIVED VACCINE

Abstract

Subunit vaccine technology has increased the safety of administering vaccines

because it does not involve the use of live or weakened viruses. Currently, it is very

expensive to produce subunit vaccines as well as to store them because they are not heat-

stable. Producing the vaccines in plants eliminates the heat-stability issue and the

contamination issue with bacterial, viral, or fungal products during production, because

the vaccinogenic plant tissue can be administered raw, dried, or in an encapsulated form;

all of these forms can be stored and shipped at room temperature. Newcastle disease virus

(NDV) is a common poultry virus from the family Paramyxoviridae, and causes severe

economic losses to farmers and governments worldwide. The current method of

vaccinating poultry is to use conventionally derived NDV vaccines. These vaccines are

produced using embryonated eggs but there are limitations with this process. One way to

overcome the limitations of conventionally made NDV vaccine is to produce the protein

in a plant cell. Dow AgroSciences has developed a plant-made vaccine production

system. Both conventional NDV vaccines and plant-cell derived NDV vaccines pose

different safety considerations. This study examined the potential risks associated with

conventionally derived NDV vaccine and plant-cell derived NDV vaccine through a

qualitative risk assessment process. The risks were characterized based on the potential

exposures to the poultry being vaccinated (target organisms), to humans administering the vaccine, and to non-target birds. The qualitative assessment illustrated that there are fewer effects and contamination issues associated with the use of the plant-cell derived NDV vaccine. A mathematical estimate of risk to target poultry, humans, and non-target birds could not be characterized because there is currently no quantitative information available. However, a qualitative risk assessment can be used as a starting point to determine where information is needed to conduct a quantitative risk assessment, but should not replace a quantitative risk assessment.

Introduction

The new technology platform of plant-based pharmaceuticals includes the ability to produce vaccines in addition to pharmaceuticals and other biologics. Subunit vaccines are made up of specific macromolecules that induce a protective immune response against a pathogen. Subunit vaccine technology has increased the safety of administering vaccines because it does not involve the use of live or weakened viruses. Currently, it is very expensive to produce subunit vaccines as well as to store them because they are not heat-stable. Because the vaccines are not heat-stable, this limits where they can be sent for use, resulting in less availability to developing countries where vaccines are needed. Producing the vaccines in plants eliminates the heat-stability issue because the vaccinogenic plant tissue can be administered raw, dried, or in an encapsulated form; all of these forms can be stored and shipped at room temperature (Sala et al. 2003). The risk of contamination with animal pathogens during production is also eliminated (Walmsley et al. 2000).

Recombinant proteins can be transformed into plant-cell cultures. Plant cell suspension cultures produce recombinant proteins more rapidly than transgenic plants because testing time to ensure transgene expression stability and testing against adverse phenotypic changes in the host plant can take up to two years, whereas in plant cells the development and testing is much shorter. Cell cultures also are contained in buildings rather than produced in the open environment thereby, reducing the risk of contamination to non-transgenic plants (Twyman et al. 2003).

Tobacco plant cell cultures are grown by disturbing the friable callus tissue to create a homogeneous suspension of single cells. The cultures are maintained in conventional microbial fermentation vats, and the system allows for fairly clean cultures (free of contamination) along with fairly straightforward purification protocols (Ma et al. 2003). Plant-based pharmaceuticals would be useful in the case of Newcastle disease vaccines because many of the conventionally derived Newcastle disease vaccines deteriorate after storage for one or two hours at room temperature, making them unsuitable for use in areas where the vaccine may need to be transported for hours, or in some cases, days at ambient temperatures (Grimes 2002).

Newcastle disease virus (NDV) is a common poultry virus from the family Paramyxoviridae. This disease causes severe economic losses to farmers and governments worldwide. In late 2002, exotic NDV spread from California to Nevada and Arizona before it could be contained. About 3.5 million commercial and backyard poultry including chickens, geese, peacocks, pigeons, and turkeys were euthanized to

stop the disease from entering other states. More than $104.5 million have been spent by state and federal task forces to try to contain and eradicate exotic NDV (Durham 2003).

The current method of vaccinating poultry is to use conventionally derived NDV vaccines. These vaccines are produced using embryonated eggs, and the virus is either killed or attenuated to induce an immune response and give protection to the poultry after administration of the vaccine. Many NDV vaccines deteriorate after storage for one or two hours at room temperature. This makes them unsuitable for use in areas where the vaccine may need to be transported for hours or days at warm temperatures. Even with the use of the live virus vaccines there is still a risk of disease to the poultry producer.

One way to overcome the limitations of conventionally made NDV vaccine is to produce the protein in a plant cell. Dow AgroSciences has developed a plant-made vaccine production system based on the use of a *Nicotiana tobacum*-derived plant cell line from a seedling of the cultivar Bright Yellow 2 (BY-2), originally from Japan. Dow AgroSciences' initial product using the recombinant NT-1 or BY-2 cell lines is the ND vaccine. The cell lines were generated by *Agrobacterium* transformation and engineered to express hemagglutinin-neuraminidase gene (*hn*) of NDV and a phosphinothricin N-acetyltransferase (*pat*) gene, which is the selectable marker (Dow AgroSciences 2004).

Risk assessment is a formal discipline that objectively evaluates risk in a manner in which assumptions and uncertainties are clearly defined (NRC 1983). The process of risk assessment flows in a logical stepwise fashion that includes the following five steps: (1) problem formulation; (2) hazard identification; (3) dose-response relationships; (4) exposure assessment; and (5) risk characterization. Hazard and dose are considered in

relation to exposure to determine risk or what additional information is needed to calculate or refine risk estimates (NRC 1983).

Conventional NDV vaccine poses different safety considerations than plant-cell derived NDV vaccine. Both NDV vaccines are produced in closed systems; however, delivery and potential contamination of the vaccines are different. The objective of this study was to examine the potential risks associated with conventionally derived NDV vaccine and plant-cell derived NDV vaccine through a qualitative risk assessment process. The process can be used to identify potential risks of each system and identify uncertainties where more information needs to be gathered to conduct a quantitative risk assessment.

Materials and Methods

Problem formulation

A qualitative risk assessment was conducted to characterize the potential risks associated with plant-cell derived NDV vaccine compared to conventionally derived NDV. The stressors (or hazards) were the NDV vaccines and the receptors were poultry, humans, and non-target birds. Therefore, the risks were characterized based on the potential exposures to the poultry being vaccinated (target organisms), to humans administering the vaccine, and to non-target birds. Both plant-cell derived NDV and conventionally derived NDV vaccines were compared qualitatively based on identified deleterious effects and exposures.

Analysis: Effects and Exposure

    Conventional NDV Vaccine. In the US, conventionally derived NDV vaccines that are sold to people or companies raising poultry, and the vaccines have been approved by the USDA Center for Veterinary Biologics (CVB) and are considered pure, safe, potent, and effective (CVB 2006). The CVB is responsible for information regarding any contamination issues or problems with any of the vaccines they receive for certification. Therefore, any conventional NDV vaccine that is sold commercially is considered safe with no issues regarding its purity, potency, and efficacy (CVB 2006). This does not mean, however, that the conventional vaccines will not cause viral symptoms in the poultry after administration.

    The mission of the USDA is to ensure that animal immunobiologics are in compliance with the Virus-Serum-Toxin Act; therefore any adverse event associated with an immunobiologic is reported to the CVB or the manufacturer. According to the CVB, an adverse event is any undesirable occurrence after the use of an immunobiological product, including illness or reaction, whether or not the event was caused by the product (CVB 2006). An immunobiologic is defined as a biological product that modulates the immune system for the prevention, treatment, or diagnosis of disease. Veterinary immunobiologics used to prevent disease include vaccines or toxoids, which stimulate an animal to produce antibody against specific organisms or substances (CVB 2006).

    Products intended for animal use must be tested for purity, safety, potency, and efficacy before they can be marketed. As a further check on the manufacturer's quality control, the CVB regularly tests randomly selected lots of all products in their laboratory.

The frequency of an adverse event occurring with veterinary vaccines is not easily estimated. According to the CVB, reporting an adverse event is voluntary to either the CVB or the manufacturer, and the reporting rate may not reflect the incidence rate. The relationship between the reporting rate and the incidence rate may vary by type and severity of event, species, manufacturer, and from one month to the next. Also, the receipt of a report to the CVB does not necessarily imply that the product caused an adverse event, or that the event even occurred. Despite these issues with reporting an adverse event, the reporting rate may be used to estimate the minimum incidence under appropriate condition (CVB 2006). Adverse event reports in avian species are rare. From 1999-2005 the CVB has had two adverse event reports that involved birds, neither of which involved NDV vaccine (Patricia Foley, USDA CVB, personal communication).

There are three NDV groups, which are characterized by the severity of the disease in chickens, and the mean death time of inoculated chicken embryos. Velogenic strains are highly virulent to birds of all ages. Chickens infected with these strains develop severe disease symptoms, including hemorrhagic intestinal lesions and acute respiratory and nervous disorders. A minimal lethal dose of an intravenous pathogenicity index of 2.0-3.0 of these strains induces mortality in embryos in less than 60 hours (Gallili et al. 1998). Mesogenic strains are less virulent and usually produce a mild disease and low mortality in young chickens, including coughing and loss of weight and egg quality and production. Lentogenic strains are the least virulent, showing almost no sign of the disease or very mild symptoms. There may be mild respiratory signs and impaired appetite and even a drop in egg production, but deaths are rare.

The virus is shed during incubation and for a varying, but limited, period when the chick is returning to a healthy state after illness. The virus is present in exhaled air, respiratory discharges, feces, eggs laid during clinical disease, and all parts of the body during acute infection and at death. NDV infects chickens of all ages. The disease spreads by direct contact by any of the above-mentioned sources and can be transmitted to poultry farms by other free-flying birds (Degefa et al. 2004).

The clinical signs in the chicken or other poultry depend on whether or not the virus is neurotropic or viscerotropic. Neurotropic viruses result in respiratory and nervous signs. Viscerotropic viruses, which predominate worldwide, cause respiratory signs with peracute disease, water-greenish diarrhea, and swelling of the tissues of the head and neck. Mortality from neurtropic and viscerotropic viruses depends on the virulence of the virus, species infected, environmental conditions, and the immune status of the flock. In general, mortality is higher in young flocks, but 100% mortality may occur in adult flocks as well (The Merck Veterinary Manual 2003).

Today, the NDV vaccine in poultry is produced using embryonated eggs, and is either attenuated (weakened) live virus or killed virus, which stimulates an immune response to induce protection from the virus. Proper administration of a high-titered vaccine is essential for induction of an immune response. Live-virus vaccines such as the lentogenic strains, B1 and LaSota, are the most common and administered in drinking water or spray. In the US and several other countries, disease control officials use import restrictions and eradication methods to prevent establishment of the viscerotropic type of the disease; other countries depend on vaccination (The Merck Veterinary Manual 2003).

There are possible effects on target poultry, humans, and non-target birds with the use of conventional NDV vaccines. Possible effects could include the ability of the vaccine to cause disease, and contamination of the vaccine with other animal diseases and toxins based on the method of production in fertilized eggs. Effects on other non-target organisms are not being considered in this risk assessment because NDV is an avian paramyxovirus Type-1 and mostly affects chickens. There are eight other types of paramyxoviruses, which differ in their host ranges and clinical signs, but they only affect avian species. Therefore, there is no reason to address any effects on other non-target organisms.

Effects on target poultry from administration of NDV vaccine include possible manifestation of Newcastle disease. Live virus vaccines are the most common NDV vaccines given to poultry. Live vaccines can replicate in host cells and manifest some of the signs of the disease. The severity of this reaction depends on the vaccinal strain used and the presence of concurrent infection with other pathogens (Westbury et al. 1984).

Effects on humans from unintended inoculation exposure to the NDV vaccine include mild flu-like symptoms, conjunctivitis, and laryngitis (Berkelman 2003). Further as a whole-tumor vaccine, mild headache and mild fever can occur on the day of vaccination. Itching, swelling, and erythema at injection sites are the most commonly reported side effects following injection of NDV-infected whole-cell vaccines. Whole-cell vaccines are made using whole tumor cells infected with NDV and currently are not used as an NDV vaccine in poultry. Researchers are interested in NDV because it replicates itself more quickly in human cancer cells than in most normal human cells and

it can kill the host cells. For these reasons, the virus is being studied as a treatment for cancer in humans (National Cancer Institute 2006).

Exposure routes in which potential effects could occur include the dosage regimen or administration to poultry and the effect on humans from each regimen for both plant-cell derived and conventional NDV vaccines. Conventional NDV vaccines can be administered via spray, injection, or water. Tiny droplets of the vaccine after spray administration to the chickens could enter into mucous membranes of the individual giving the vaccine. Accidental injection of a full dose of the vaccine meant for the chicken could also occur and cause a deleterious effect to the individual administering the vaccine. The exposure and effects to poultry from conventional NDV vaccine for all exposure routes include manifesting symptoms of the disease to death after administration.

Effects on non-target birds from exposure to the vaccine would be associated with incidental exposure to the vaccine. Tolerization (a specific unresponsiveness to self antigens) in poultry and other non-target birds is not likely from a non-sufficient dose because it is routine to administer fractions of a dose per bird using mass administration routes.

The vaccine can be administered in the water and may spread into the environment via water, but its environmental stability and photo stability are unknown. The drinking water systems for most chicken facilities in the US are low-pressure systems that have backflow preventers to limit the water going back into the lines. Flow is usually cut off during vaccination to make sure the birds consume the vaccine. A

broken or leaky water line could cause a flood, potentially leaking outside of the chicken house and then potentially exposing a susceptible non-target bird. The potential of this, however, is very unlikely (Michel Carr, USDA CVB, personal communication). Another possible exposure route to non-target birds is contact with infected chickens and the disease spreading from bird to bird. However, NDV virus is an avian paramyxovirus Type-1 and affects mostly chickens. There are eight other types of paramyxoviruses, which differ in their host ranges and clinical signs. Most birds are considered susceptible to paramyxovirus Type-1 infection but not all show clinical signs. Conventional NDV vaccines can be shed into the environment from the poultry farm or facility or from the poultry itself and spread in non-target birds by replication.

Plant-cell Derived NDV Vaccine. As stated above, plant-cell derived NDV vaccine has been developed by Dow AgroSciences and was approved by the CVB in January 2006 (USDA APHIS 2006). Approval by the CVB, denotes the vaccine as pure, safe, potent, and effective.

The cell line, NT-1, used by Dow AgroSciences has been safely used in research laboratories and propagated for decades. The plant-cell line also lacks nicotine and cannot regenerate into whole plants from the culture. The final product is a non-replicating subunit vaccine for the immunization of healthy chickens, and it cannot propagate in the target host. This vaccine is administered through several methods, such as subcutaneously, intranasally, orally, or *in ovo* (Dow AgroSciences 2004).

The master-seed recombinant cell line was designated as CHN-18, and more than 6,000 one-day old chicks have been vaccinated with CHN-18. The chicks did not show

any abnormalities, and because the vaccine is non-replicating it is not expected to create any adverse reactions with the virulent form of NDV. Because it is non-replicating, this virus does not pose any pathogenicity or safety risks to non-target animals, or possible environmental shedding or spreading. These plant cells are not exposed to the open environment, and they are only alive during production in a bioreactor (Dow AgroSciences 2004).

The vaccine will most likely be administered via the feed, either as a top-dress or incorporated into the food. No adverse systemic or local reactions have been observed upon administration of the vaccine, and there is no evidence that any type of immunosuppression would occur with plant-cell derived NDV (Dow AgroSciences 2004). Plant-cell derived NDV is non-replicating and could not revert to virulence. The NT-1 cell line has no known pathogenicity, and it is genetically stable over 40 passages of the master seed. The NT-1 cell line is also phenotypically stable over 40 passages. The vaccine does not pose any risk of overdosing because it is non-replicating and there is no concern of pathogenicity of the vaccine. There are no safety issues of plant-cell derived NDV to non-target animals with regards to susceptibility and virulence (Dow AgroSciences 2004). The exposure and effects of plant-cell derived NDV vaccine to poultry via top-dressing the feed is unknown. However, it is known that plant-cell derived NDV vaccine is a non-replicating subunit vaccine that poses no harm to poultry, but the question remains of the effect if the dose is not sufficient to initiate an immune response in the poultry after the poultry are administered the vaccine through top-dressing the feed.

Human exposure to either vaccine (conventional NDV or plant-cell derived NDV) could occur during manufacture, transport, or administration to the animal. Accidental exposure to the vaccine during administration would be the most likely means of direct human exposure. The exposure to humans from plant-cell derived NDV vaccine that is administered via top-dressing the feed is unknown. However, unlike the conventional vaccine, the plant-cell derived vaccine has no known pathogenicity or virulence to humans (Dow AgroSciences 2004).

There are no effects to non-target birds from plant-cell derived NDV vaccine, because as a non-replicating subunit vaccine it does not pose any pathogenicity or safety risks to non-targets birds. There is also no virulence associated with the vaccine to non-target birds. There is the possibility of the plant-cell derived vaccine on food to "escape" the chicken facility and be released into the environment; however, because it is non-replicating, there is no potential of it spreading into the environment and therefore having a virulent and pathogenic effect to non-target birds. Shed or spread is a concern with most biotechnology derived products but plant-cell derived NDV is a non-replicating vaccine and only the HN protein is present from the vaccine, so there is no concern of shed or spread into the environment. There is no risk of gene transmission or recombination of plant-cell derived NDV vaccine since the plant cells are only live during production in a contained bioreactor. The live plant cells are never in the open environment. The plant cells are not pathogens so there is no specific host or host range. In an event of accidental release of the live plant cells in the environment, the plant cells would not

survive for a long period of time because they require growth media to survive. They also cannot regenerate into a whole plant.

Results and Discussion

The qualitative assessment presented here illustrates the differences between plant-cell derived and conventional NDV vaccines. There are fewer effect and contamination issues associated with use of the plant-cell derived NDV vaccine (Table 1). The results show that the consideration for a cold-chain process for conventional NDV vaccines exists to maintain functionality of the vaccine. This is unlike plant-cell derived NDV vaccines in which the cold-chain is eliminated from the delivery process. Contamination issues associated with conventional NDV vaccine include potential bacterial, viral, and fungal contamination of the vaccine during the production process. Contamination of the vaccine is possible due to production in SPF eggs and its potential exposure to bacterial, viral, and fungal agents and their ability to replicate within host cells. For plant-cell derived NDV vaccine, there would be no bacterial, viral, or fungal contamination issues for the host because it is a non-replicating subunit vaccine. The effects on humans, target poultry, and non-target birds from conventional NDV vaccine exist based on the virus itself and its ability to infect, and contamination of the virus and the potential to cause a symptom from a bacterial, fungal, or viral infection not associated with NDV. Plant-cell derived NDV vaccines do not pose any deleterious effects to human, target poultry, or non-target birds because of its non-replicating property and inability to be pathogenic and virulent (Table 1).

A mathematical estimate of risk to target poultry, humans, and non-target birds cannot be characterized at this time because there is currently no quantitative information available. However, a qualitative risk assessment provides a starting point to communicate the risks associated with both vaccine systems and clearly identifies where more information is needed. Qualitative assessment can be used as a regulatory guide based on the exposures outlined and the potential harm or risk to determine an overall rating or conclusion of risk for decision making.

There are uncertainties associated with this qualitative assessment. The uncertainties associated with exposure routes for both conventional NDV vaccine and plant-cell derived NDV include the administration to poultry and the effect to humans from each regimen. There are a few methods of delivery associated with conventional NDV vaccines, and it is unknown if the vaccine potentially "escapes" what its effects would be on non-target poultry, and how likely it is for this to occur. The stability of both plant-cell derived NDV and conventionally derived NDV in the environment is unknown; therefore, there is an uncertainty associated with the vaccine's exposure to non-target birds. The exposure and effects of plant-cell derived NDV to poultry via top dressing the feed is an uncertainty. It is known that plant-cell derived NDV is a non-replicating subunit vaccine that poses no harm to poultry, but the question remains of the effect if the dose is not sufficient to initiate an immune response in the poultry after the poultry are administered the vaccine through top-dressing the feed.

Both plant-cell derived NDV and conventionally derived NDV vaccines have strengths and shortcomings that contribute to the cost-benefit aspect of using either

system. The cost-benefit analysis can be based on the differences in safety of each vaccine, as well as actual costs to develop and administer the vaccines. The overall cost of using plant-cell systems versus conventional vaccine production systems needs to be assessed from all processing ends to the final delivery of the vaccine for any conclusion determining the most cost effective process.

The financial cost of drug production is most influenced by downstream processing and manufacturing under good manufacturing practices (GMP). Growing yeast and bacteria is inexpensive, but the cost increases when GMP is applied, making the overall technology costs change. As far as plant-based vaccines are concerned, it is unknown if the downstream process and quality control costs would be significantly different from conventional vaccine production (Kirk and Webb 2005).

The formulation and production of human plant-based vaccines with non-generally recognized as safe (GRAS) substances will require a complete battery of pre-clinical assessments consistent with other vaccine trials. This is relevant for those attempting to make a plant-based vaccine using plant cell cultures, plant hairy root cultures, or other plant tissues that do not have a long history of use in the common diet (Kirk and Webb 2005). This concept could be applied to animal plant-based vaccine production as well, which points out shortcomings with the development of any plant-based vaccine or pharmaceutical.

To make the production of plant-based vaccines financially feasible, the expression and processing methods need to be improved. Although the costs of goods and "run costs" may be expensive for plant-based vaccine production, downstream

distribution and administration may be greatly reduced by the elimination of refrigeration and needles, and reduction in product waste (Kirk and Webb 2005).

The shortcomings, as far as financial costs are concerned, for conventional vaccine production is the development time to produce enough vaccine. It takes an extensive amount of time and the capacity is limited to SPF eggs (Parker 2005). Specific-pathogen-free eggs are derived from SPF flocks. These flocks are bred in isolated buildings and supplied with filtered air and frequent testing to ensure microbiological integrity of the flock (Spradbrow 1993). These flocks are very expensive to maintain, and therefore SPF eggs are very expensive. SPF eggs can also be easily contaminated by bacteria, resulting in a loss of product.

The USDA uses a different method of qualitative assessment. The rating system used by the USDA determines the risk of plant-cell derived NDV based on safety characteristics of the vaccine. The risk assessment uses qualitative assumptions based on studies, and then gives a likelihood rating, a consequence rating, and a degree of certainty rating for each and then multiplies the assigned values to reach an overall risk rating to produce a "quasi" quantitative assessment (USDA APHIS 2005). The risk characterization integrates the results of the hazard identification and the release assessment into a risk statement that includes a likelihood rating, a consequence rating, a risk rating and a discussion of the risk. The likelihood and consequence rating is qualified by a degree of certainty rating that includes a justification for the rating. The risk rating is based upon the likelihood, consequence, and degree of certainty ratings. The numerical value ratings were derived from the importance placed on the rating for

each category. They are weighed to place emphasis on the severity of the expected risk.

The expected risk is then calculated by multiplying the numerical values: [(likelihood) x

(degree certainty)] x [(consequence) x (degree of certainty)] = risk rating. There are a

total of 81 rating combinations because the likelihood rating can have a rating of low,

medium or high, the consequence rating can have a rating of low, medium, or high. The

severity of the risk reflects the professional judgment of CVB, APHIS. A low rating is an

acceptable risk with very little concern associated with the proposed product. A medium

rating is an unacceptable risk rating with moderate concern associated with the proposed

product and mitigative procedures are proposed or the proposed product is denied.

Finally, a high rating is an unacceptable risk with major concerns with the proposed

product and the product proposal is denied.

Using a rating system such as the procedure used by USDA/APHIS, brings into

question the accuracy of the final risk rating, which appears to be quantitative, but in

reality is not. The approach does not include a separate zero or "negligible" qualitative

value. With this method, risk is always rated "high" if the consequence component is

rated "critically important," even if the exposures and adverse effects from exposure are

all zero or negligible (Cox et al. 2005). There are no calculations using endpoint values

such as no effect levels (NOELs) or LD50s. A quantitative risk assessment would reveal

the uncertainties to the regulatory agencies and likely save time and direct the agencies

where mitigation needs to take place. A qualitative risk assessment can be used as a

starting point to determine where information is needed to conduct a quantitative risk

assessment, but should not replace a quantitative risk assessment. Qualitative risk

methods using ordered categorical labels such as "high", "medium," and "low" can potentially simplify risk assessment input requirements used to inform risk management decisions (Cox et al. 2005). However, qualitative risk assessments often do not provide sufficient information to discriminate accurately between quantitatively small and quantitatively large risks.

There are potential risks associated with NDV vaccines to humans, poultry, and non-target birds. These risks range from conjunctivitis in humans to clinical signs in non-target poultry to possible death in poultry from administration of conventional NDV vaccines. Alternatively, these risks may outweigh the economic loss of a large flock of chickens. However, it is apparent that there are fewer deleterious effects with plant-cell derived NDV vaccines when compared to conventionally derived NDV vaccines. Producing subunit biologics in plant-cells eliminates many of the contamination and deleterious effects associated with conventionally produced vaccines. This technology platform may offer a method of overcoming most effects caused by conventional production of vaccines as well as eliminate the cold-chain process making vaccines more assessable to areas without cold storage systems.

Table 10. Conventional NDV Vaccine Compared to Plant-cell Derived
     NDV Vaccine.

	Conventional NDV	Plant-cell derived NDV
Cold-chain Considerations	Yes	No
Bacterial Contamination	Yes	No
Fungal Contamination	Yes	No
Viral Contamination	Yes	No
Effects on Humans	Yes	No
Effects on Target Poultry	Yes	No
Effects on Non-Target Birds	Yes	No

CHAPTER 5

CONCLUSION

Quantitative human-health and ecological risk assessments were conducted for three pharmaceutical proteins expressed in maize kernels. A comparative qualitative risk assessment on plant-cell derived and conventionally derived Newcastle disease (NDV) vaccine was also conducted. The pharmaceutical proteins are all characteristically different from each other; therefore, risk assessments were done to demonstrate the need to evaluate plant-based pharmaceuticals on a case-by-case basis.

The human-health risk assessment established that human-health risks vary dramatically and depend on factors such as the specific pharmaceutical protein, protein expression, and exposure scenario. The greatest difference in risks was attributable to the differences in toxic endpoints of the proteins. Although the protein expressions and dietary consumption were assumed to be the same for all proteins, dietary risks between the proteins varied by more than 56,000 times, reflecting the toxic endpoint differences between gastric lipase (1000 mg/kg BW) and LT-B (0.016 mg/kg BW). From the findings in the risk assessment, confinement of aprotinin maize and LT-B maize would be necessary based on uncertainties regarding repeated dietary exposures and allergenicity. Also, when aprotinin is expressed at 1000 mg/kg, the risk quotients (RQ's) for all age groups would be above levels of concern for the scenario in which the transgenic maize expressing the therapeutic protein was harvested and accidentally taken to a specialty food processing facility. The specialty food processing facility makes tortilla chips, and the transgenic maize was made directly into the tortilla chips with no

dilution from non-transgenic maize and the protein was not denatured.   The scenarios

applied to this risk assessment did not incorporate USDA permit guidelines (USDA

APHIS 2006). Had those guidelines been taken into account with this risk assessment

(e.g., distance between fields, planting time, and fallow zone), exposure and subsequent

dietary risks for all scenarios would be much less.

The ecological risk assessment for plant-based pharmaceuticals was conducted

for four receptor species used as surrogates for a wider range of species. The same three

proteins used for the human-health risk assessment were used for the ecological risk

assessment. Protein expression was considered the same for all three proteins, and,

currently there is considerable uncertainty with that assumption. When comparing the

RQ's of all the proteins for all the ecological receptors, the RQ's were highest for LT-B.

The RQ's under all the RQ percentiles for LT-B were highest for vole and then quail,

both exceeding values of 1.0. Gastric lipase had the lowest RQ values for all the

ecological receptors. When examining gastric lipase alone, vole, had the highest RQ

values for all three percentiles (50th, 75th, 90th). The RQ's for aprotinin were between the

values for LT-B and gastric lipase, and, when examining the ecological receptors within

aprotinin, vole had the highest RQ values. The greatest contributor to variance in total

exposure was protein expression. The ecological risk assessment demonstrated that risks

will vary between species and between proteins, based primarily on differences in toxic

endpoint and consumption rates. It also shows the utility of probabilistic, quantitative

risk assessment methodologies and supports the human dietary risk assessment by

demonstrating the importance of assessing risks from plant-based pharmaceuticals on a case-by-case basis.

The comparative qualitative risk assessment compared potential deleterious effects and exposures associated with plant-cell derived NDV vaccine and conventionally derived NDV vaccine. NDV vaccine in poultry is produced using embryonated eggs, and is either attenuated (weakened) live virus or killed virus, which stimulates an immune response to induce protection from the virus. There are possible effects on target poultry, humans, and non-target birds with the use of conventional NDV vaccines. The plant-cell derived NDV vaccine is produced in tobacco cell line NT-1, and the final product is a non-replicating subunit vaccine for the immunization of healthy chickens. The plant-cell derived NDV vaccine lacks nicotine and cannot regenerate into whole plants from the culture and it cannot propagate in the target host. The risks were characterized based on the potential exposures to the poultry being vaccinated (target organisms), to humans administering the vaccine, and to non-target birds. Plant-cell derived NDV vaccines do not pose any deleterious effects to humans, target poultry, or non-target birds because of its non-replicating property and inability to be pathogenic and virulent. This qualitative risk assessment can be used as a starting point to determine where information is needed to conduct a quantitative risk assessment, but should not replace a quantitative risk assessment. It was concluded that there are fewer effects and contamination issues associated with the use of the plant-cell derived NDV vaccine. Also, producing subunit biologics in plant cells eliminates many of the contamination and deleterious effects associated with conventionally produced vaccines.

REFERENCES CITED

Adamcakova-Dodd, A., Thorne, P., Ludewig, G. 2004. Human health risk assessment of plant-made pharmaceuticals. Final version. University of Iowa. Iowa City, Iowa.

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.

Alabama Land Trust. 2005. http://www.allandtrust.org/. Piedmont, AL.

Anonymous. 2001. Aprotinin. Monograf UBAT 173. August: 1-13. Malaysia.

ANZFA. 2002. Australia New Zealand Food Authority. Final Assessment Report. Lipase from genetically modified *Aspergillus oryzae.*

Aprotinin Patent. 1998. Commercial production of aprotinin in plants. U.S. Patent 5,824,870 awarded to Baszczynski et al.

Australia New Zealand Food Authority (ANZFA). 2002. Lipase from genetically modified *Aspergillus oryzae.* Application (A435). 21 p.

Bayer Pharmaceuticals Corporation. 2003. Trasylol® aprotinin injection. Product Label. West Haven, CT.

Basque, Jean-Rene and Menard, Daniel. 1999. Establishment of culture systems of human gastric epithelium for the study of pepsinogen and gastric lipase synthesis and secretion. Microscopy Research and Technique. 48: 293-302

Beierlein, W., Scheule, A.M., Antoniadis, G., Braun, C., Schosser, R. 2000. An immediate, allergic skin reaction to aprotinin after reexposure to fibrin sealant. Transfusion. 40: 302-305.

Bioprotein Technologies. 2005. Paris, France. http://www.bioprotein.com/gb/faq.htm#Séquence_1

Carr, Michel. USDA CVB. Personal Communication Email. 10/24/2006.

CDC. 2005. U.S. Center for Disease Control. Vaccine Safety. http://www.cdc.gov/nip/publications/pink/safety.pdf. Atlanta, GA.

CFR. 2004. Code of Federal Regulations. Animals and animal products. 9: 101-106.1. Office of the Federal Register National Archives and Records Administration. Washington, D.C.

CVB. 2006. U.S. Department of Agriculture APHIS Center for Veterinary Biologics. http://www.aphis.usda.gov/vs/cvb/index.html. Ames, IA.

Chikwamba, R., McMurray, J., Shou, H., Frame, B., Pegg, S., Scott, P., Mason, H., Wang, K. 2002. Expression of a synthetic E. coli heat-labile enterotoxin B sub-unit (LT-B) in maize. Molecular Breeding 10: 253-265.

Chikwamba, R.K. et al. 2003. Localization of a bacterial protein in starch granules of transgenic maize kernels. Proceedings of the National Academy of Sciences. 100: 11127-11132.

Christensen, P.J., Misra, M.K., Rai, S., Shyy, Y-Y, Wolt, J.D. 2004. Confined production processes for non-food corn. Biosafety Institute for Genetically Modified Agricultural Products, Iowa State University, Ames IA. 81 p.

Coenen, T.M.M., Aughton, P., Verhagen, H. 1997. Safety evaluation of lipase derived from *Rhizopus oryzae*: summary of toxicological data. Food and Chemical Toxicology 35: 315-322.

Cook, J. 2003. Biotechnology: cause and consequence of change in agriculture. In A. Eaglesham, S. Ristow, and R. Hardy (eds.), NABC Report 1, Biotechnology: Science and society at a crossroad. National Agricultural Biotechnology Council, Ithaca, New York. Pp. 39-52.

Columbia Encyclopedia. 2005. Sixth Edition. http://www.encyclopedia.com/html/01/orphandru.asp.

Costello, J.M., Backer, A. de Hoyos, Binns, H.J., Mavroudis, C. 2003. Aprotinin reduces operative closure time and blood product use after pediactric bypass. The Annals of Thoracic Surgery. 75: 1261-1266.

Curtiss R, 3rd Cardineau G.A. 1990. Oral immunization by transgenic plants. World Patent Application WO 90/02484. Washington University (Assignee).

Degefa, T., Dadi, L., Yami, A., Mariam, K.G., Nassir, M. 2004. Technical and economic evaluation of different methods of Newcastle disease vaccine administration. Journal of Veterinary Medicine 51: 365-369.

Dow AgroSciences. 2004. Risk analysis: Newcastle disease vaccine, subunit. Indianapolis, IN. submitted 6/15/04.

Dlugosz, J. et al. 1988. Feedback regulation of stimulated pancreatic enzyme secretion during intraduodenal perfusion of trypsin in man. European Journal of Clinical Investigation 18: 267-272.

Durham, S. 2003. An END for exotic Newcastle disease virus? USDA ARS. Beltsville, MD. http://www.ars.usda.gov/is/AR/archive/oct03/virus1003.htm.

Ellenberg, S.S., Braun, M.M. 2002. Monitoring the safety of vaccines: Assessing the risks. Drug Safety 25: 145-152.

Ellenberg, S.S., Chen, R.T. 1997. The complicated task of monitoring vaccine safety. Journal of U.S. Public Health Reports 112: 10-20.

EMEA. 2003. The European agency for the evaluation of medicinal products pre-authorization evaluation of medicines for human use. Press release 37th meeting of the committee for orphan medicinal products.

FAO. 2001. Food and Agriculture Organization. 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.

Fauli, A., Gomar, C., Campistol, J.M., Alverez, L., Manig, A.M., Marute, P. 2005. Kidney-specific proteins in patients receiving aprotinin at high-and low-dose regimens during coronary artery bypass graft with cardiopulmonary bypass. European Journal of Anesthesiology. 22: 666-671.

Fischer, R., and Emans, N. 2000. Molecular farming of pharmaceutical proteins. Transgenic Research 9: 279-299.

Fischer, R., Twyman, R.M., Schillberg, S. 2003. Production of antibodies in plants and their use for global health. Vaccine 21: 820-825.

Flood, M.T., and Kundo, M. 2003. Safety evaluation of lipase produced from *Rhizopus oryzae*: summary of toxicological data. Regulatory Toxicology and Pharmacology 37: 293-304.

Foley, Patricia. USDA, CVB. Personal Communication Email. 10/24/06.

Freese, Bill. 2002. Manufacturing drugs and chemicals in crops: biopharming poses new threats to consumers, farmers, food companies and the environment. Genetically Engineered Food Alert. http://www.foe.org/biopharm/.

Freese, B. 2005. Biopharming: case study of corn-produced aprotinin. Friends of the Earth, for Genetically Engineered Food Alert. http://www.foe.org/biopharm/.

Gallili, G.E., Ben-Nathan, D. 1998. Newcastle disease vaccine. Biotechnology Advances 16: 343-366.

Gendel, S. 1998. The use of amino acid sequence alignments to assess potential allergenicity of proteins used in genetically modified foods. Advances in Food and Nutrition Research 42: 45-62.

Giddings, G., Allison, G., Brooks, D., Carter, A. 2000. Transgenic plants as factories for biopharmaceuticals. Nature Biotechnology 18: 1151-1155.

Gomord, V., Sourrouille, C., Fitchette, A-C., Bardor, M., Pagny, S., Lerouge, P., Faye, L. 2004. Production and glycosylation of plant-made pharmaceuticals: the antibodies as a challenge. Plant Biotechnology Journal 2: 83-100.

Greenough, R.J. et al. 1996. Safety evaluation of a lipase expressed in *Aspergillus oryzae.* Food Chemical Toxicity 34: 161-166.

Grimes, S.E. 2002. A basic laboratory manual for the small-scale production and testing of I-2 newcastle disease vaccine. FAO Regional Office for Asia and the Pacific (RAP). Bangkok, Thailand. http://www.fao.or.th.

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

Iowa State University. 2003. Enterotoxin B. ISU, Ames IA.

Jaquiss, R.D., Ghanayem, N.S., Sacharisen, M.C., Mussatto, K.A., Tweddell, J.S., Litwin, S.B. 2002. Safety of aprotinin use and re-use in pediatric cardiothoracic surgery. Circulation 106 (12 Suppl 1): 190-194.

Kidwell, J.L., Tomerline, J.R., Peterson, B.J. 2000. DEEM[TM] Dietary Exposure Evaluation Model. Novigen Sciences, Inc. Washington, D.C.

Kirk, D.D., McIntosh, K., Walmsley, A.M., Peterson, R.K.D. 2005. Risk analysis for plant-made vaccines. Transgenic Research 14: 449-462.

Kirk, D.D., and Webb, S.R. 2005. The next 15 years: taking plant-made vaccines beyond proof of concept. Immunology and Cell Biology 83: 248-256.

Lamphear, B.J. et al. 2002. Delivery of subunit vaccines in maize seeds. Journal of Controlled Release 85: 169-180.

Ma, K-C., Drake, Pascal M.W., Christou, P. 2003. The production of recombinant pharmaceutical proteins in plants. Nature Reviews Genetics 4: 794-805.

Ma, K-C., Barros, E., Bock, R., Christou, P., Dale, P.J., Dix, P.J., Fischer, R., Irwin, J., Mahoney, R., Pezzotti, M., Schillberg, S., Sparrow, P., Stoger, E., Twyman, R.M. 2005. Molecular farming for new drugs and vaccines. Current perspectives on the production of pharmaceuticals in transgenic plants. EMBO Reports 6 593-599.

Mangano, D.T., Tudor, J.C., and Dietzel, C. 2006. The risks associated with aprotinin in cardiac surgery. New England Journal of Medicine 354: 353-365.

Marshall, R.R. 1988. Study to evaluate the chromosome damaging potential of SP 400 by its effects on cultured human lymphocyte using an *in vitro* cytogenetics assay. Microtest Research Ltd. Study number NOD 4/HLC. Microtest Research Ltd, York.

Maugh II, T. 2006. Multiple risks of surgery drug seen. The Los Angeles Times. January 26, 2006.

McGarvey, P.B, Hammond, J., Dienelt, M.M., Hooper, D.C., Fu, Z.F., Dietzschold, B., Koprowski, H., Michaels, F.H. 1995. Expression of the rabies virus glycoprotein in transgenic tomatoes. Biotechnology 13: 1484-1487.

McNeil Pharmaceutical. 1992. Pancrease brand of Pancrelipase. McNeilab, Inc Spring House, PA.

Meristem Therapeutics. 2005. Merispase® Product label. Clermont-Ferrand, France. http://www.meristem-therapeutics.com/sommaire_en.php3.

Milano, C.A., Patel, V.S., Smith, P.K., Smith, M.S. 2002. Risk of anaphylaxis from aprotinin re-exposure during LVAD removal and heart transplantation. The Journal of Heart and Lung Transplantation. 21: 1127-1130.

Millar, G.G., Hirst, T.R., Snider, D.P. 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 Immunology 69: 3476-3482.

Nap, J-D., Metz, P.L.J., Escaler, M., Conner, A.J. 2003. The release of genetically modified crops into the environment: Part 1. Overview of current status and regulations. The Plant Journal 33: 1-18.

NRC. 1983. National Research Council. Risk Assessment in the Federal Government: Managing the Process. National Academy Press. Washington, DC.

Pedersen, P.B. 1988. SP 400 (batch no. LAJR 009 + 012): testing of mutagenic activity with *Salmonella typhimurium* TA 1535, TA 1537, TA 100, and *Escherichia coli* WP2uvrA (pKM101) in a liquid culture assay. Novo report no. 5388. Novo Nordisk A/S Bagsvaerd, Denmark.

Peterson, R.K.D., Arntzen, C.J. 2004. On risk and plant-based biopharmaceuticals. Trends in Biotechnology 22: 64-66.

Parker, R. 2005. DNA and Cell Based Vaccines In Development Against Pandemics. October, 22. http://www.futurepundit.com/archives/cat_pandemic_vaccines.html

Peterson, R. K.D., Hulting, A.G. 2004. A comparative ecological risk assessment for herbicides used on spring wheat: the effect of glyphosate when used within a glyphosate-tolerant wheat system. Weed Science 52: 834-844.

Pla, M., Messeguer, J., Mele, E. 2006. Gene flow from GE to conventional maize using real-time PCR. ISB News Report August 2006.

Prodigene, AproliZean TM – Recombinant aprotinin from maize, Version I, 3/2004. www.prodigene.com.

Roche Diagnostic Corporation. Version 6, 2003. Basel, Switzerland.

Rogers, K.K. 2003. The potential of plant-made pharmaceuticals. http://www.plantpharma.org/ials/index.php?id=1

Sala, F., Rigano, M. M., Barbante, A., Basso, B., Walmsley, A. M., Castiglione, S. 2003. Vaccine antigen production in transgenic plants: strategies, gene constructs and perspectives. Vaccine 21: 803-808.

Sedrakyan, A., Atkins, D., Treasure, T. 2006. The risk of aprotinin: a conflict of evidence. The Lancet 367: 1376-1377.

Sedgwick County Zoo. 2006. http://www.scz.org/animalinfo.asp?aid=80

Sigma Aprotinin. 2006. "Aprotinin: Sigma Product Information," Sigma Chemical Company. Available on Sigma's website at www.sigmaaldrich.com.

Shama, L., Peterson, R.K.D. 2004. The benefits and risks of producing pharmaceutical proteins in plants. Risk Management Matters 2: 28-33.

Spradbrow, P.B. 1993-94. Newcastle disease in village chickens. Poultry Science Reviews 5: 57-96.

Stavnsbjerg, M. 1988. Acute oral toxicity of SP 400, batch no. LAJ0002 + 0003 in rats. Novo report no. 0688. Novo Nordisk A/S Bagsvaerd, Denmark.

Tacket, C.O., Pasetti, M.F., Edelman, R., Howard, J.A., Streatfield, S. 2004. Immunogenicity of recombinant LT-B delivered orally to humans in transgenic corn. Vaccine 22: 4385-4389.

Twyman R.M., Stroger E., Schillberg, S., Christou, P., Fischer, R. 2003. Molecular farming in plants: host systems and expression technology. Trends in Biotechnology 21: 570-578.

The Merck Veterinary Manual. 2003. Newcastle disease: Introduction. Merck and Co, Inc. Whitehouse Station, NJ. Eighth Edition. http://www.merckvetmanual.com/mvm/index.jsp?cfile=htm/bc/203800.htm.

The Washington Times. 2004. Dow Chemical to make vaccines from genetically engineered plants. January 15, 2004. http://www.pharmacytimes.com/printerfriendly_newsfeed.cfm?ID=7852.

Walmsley A.M., Arntzen C.J. 2000. Plants for delivery of edible vaccines. Current Opinion in Biotechnology 11: 126-129.

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.

Westbury, H.A., Parsons, C. and Allan W.H. 1984. Comparison of the residual virulence of Newcastle disease vaccine strains V4, Hitchner B1 and La Sota. Austrailian Veterinary Journal. 61: 47-49.

Weltzin, R. et al. 2000. Parenteral Adjuvant Activities of *Escherichia coli* Heat-Labile Toxin and Its B Subunit for Immunization of Mice against Gastric *Helicobacter pylori* Infection. Infection and Immunity. 68: 2775-2782.

Williams, N.A. 2000. Immune modulation by the cholera-like enterotoxin B-subunits: from adjuvant to immunotherapeutic. Intl. J. Med. Microbiol. 290: 447-453.

Wildlife exposure factors handbook. 1993. Vol 1. EPA/600/R-93/187a. http://www.web.ead.anl.gov/ecorisk/methtool/wildlife/EFH3allometric.pdf.

Wolt, J.D., Shyy, Y.Y., Christensen, P.J., Dorman, K.S., Misra, M. 2004. Quantitative exposure assessment for confinement of maize biogenic systems. Environmental Biosafety Research. 3: 183-196.

Wolt, J.D., Wang, K., Peterson, R.K.D. 2006. Assessing risk of unintended antigen occurrence in food: A case instance for maize-expressed LT-B. Human and Ecological Risk Assessment 12: 856-870.

USDA APHIS. 2005. U.S. Department of Agriculture Animal and Plant Health Inspection Service. Risk assessment outline for use in preparing risk analyses for biotechnology-derived products. The Center for Veterinary Biologics. http://www.aphis.usda.gov/vs/cvb/lpd/RiskAssessmentOutline.pdf.

USDA APHIS. 2006. U.S. Department of Agriculture Animal and Plant Health Inspection Service. Draft guidance for APHIS permits for field testing or movement of organisms with pharmaceutical or industrial intent. http://www.aphis.usda.gov/brs/pdf/Pharma_Guidance.pdf.

USEPA. 1997. U.S. Environmental Protection Agency. Exposure Factors Handbook Volume 1 – General Factors. Washington DC.

USEPA. 1997. U.S. Environmental Protection Agency. Pesticides: Science and policy. http://www.epa.gov/pesticides/science/models_db.htm

USEPA. 2000. U.S. Environmental Protection Agency. SAP Report No. 2000-03B, September 28, FIFRA Scientific Advisory Panel Meeting, June 6-7, 2000, held at the Sheraton Crystal City Hotel, Arlington, Virginia. Sets of Scientific Issues Being considered by the Environmental Protection Agency Regarding: Session II –Mammalian Toxicity Assessment Guidelines for Protein Plant

USDA FDA. 2002. U.S. Department of Agriculture Food and Drug Administration. Draft guidance for industry, drugs, biologics, and medical devices derived from bioengineered plants for use in humans and animals. September.

USDA FDA. 2003. U.S. Department of Agriculture Food and Drug Administration. Guidance for Industry: Use in Humans and Animals, Draft Guidance. FDA Center for Biologics Evaluation and Research, Washington, D.C.

USDA. 2006. U.S. Department of Agriculture. The agricultural marketing service of the USDA in the Cattle Today Market Report. http://www.cattletoday.com/markets.htm

Vucicevic, Z., and Suskovic, T. 1997. Acute respiratory distress syndrome after aprotinin infusion. The Annals of Pharmacotherapy 31: 429-432.

Zhaopeng H., Kimura G., Yoshikawa Y., Takado K. 1998. Improvement of drug bioavailability using protease inhibitors. Review Japanese PubMed-indexed for Medline 56: 595-600.

Zhong, G.Y., Peterson, D., Delaney, D.E., Bailey, M., Wicher, D.R., Register, J.C., Bond, D., Li, C.P., Marshall, L., Kulisek, E., Ritland, D., Meyer, T., Hood, E.E., Howard, J.A. 1999. Commercial production of aprotinin in transgenic maize seeds. Molecular Breeding 5: 345-356.